



PATENTS  
Atty. Docket No. VOS-012

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Applicant:   | Oliver Bruestle   | Art Unit:    | 1632             |
| Serial No.:  | 09/581,890  | Examiner:    | Falk, Anne Marie |
| Filing Date: | August 28, 2000   | Conf. No.    | 7106             |
| Title:       | <b>Neural Precursor Cells, Method for<br/>the Production and Use Thereof in<br/>Neural Defect Therapy</b> | Customer No. | 23483            |

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**SECOND DECLARATION OF DR. OLIVER BRUESTLE UNDER 37 C.F.R. § 1.132**

Dear Sir:

I, Oliver Bruestle, declare as follows:

1. I am the inventor of the invention claimed in the above-referenced patent application.
2. I obtained my M.D. in 1989 from the University of Ulm Medical School, Germany. I worked as a resident in neuropathology at the University of Zurich, Switzerland, and as a resident in neurosurgery at the University of Erlangen-Nürnberg, Germany. I performed a postdoctoral fellowship at the National Institutes of Health, Bethesda, MD in the area of stem cell research and was group leader in neuropathology at the University of Bonn Medical Center. Since 2002 I have served as the Director of the Institute of Reconstructive Neurobiology at the University of Bonn Medical Center, Bonn, Germany. I have authored or co-authored several papers regarding

neurobiology and neural precursor cells. My curriculum vitae including a selected list of these publications is enclosed herewith as Appendix A.

3. Currently, I am the Director of the Institute of Reconstructive Neurobiology at the University of Bonn Medical Center in Bonn, Germany.

4. I am familiar with the Office Action dated December 2, 2005 and with Weiss *et al.*, U.S. Patent No. 5,980,885, cited in the Office Action.

5. My application has claims, as amended, directed to isolated, non-tumorigenic cell compositions. These cell compositions consist essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells. Other claimed cell compositions of my invention comprise neural spheres, wherein the neural spheres consist essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells. Yet other claimed cell compositions of my invention consist essentially of embryonic stem cell-derived glial precursor cells, and glial cells derived from the embryonic stem cell-derived glial precursor cells. The application also has claims directed to cell libraries and pharmaceutical compositions related to these cell compositions.

6. The claims as amended now all contain language such as a "cell composition consisting essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells," as indicated in the individual claims. A person schooled in neurology would be aware that cell compositions consisting essentially of different proportions of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would and could easily be obtained based on the disclosure in the specification. For example, a neurologist would be aware that depending on the amount of time allowed and the growth factors used

for proliferation or differentiation, neural precursor cells would eventually give rise to neuronal or glial cells and the relative proportion of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would change and evolve as the various types of cells proliferated, differentiated, and even died at different rates. In addition, based on the disclosure in the specification, a neurologist would know that these cell compositions consisted essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from these embryonic stem cell-derived neural precursor cells. Thus, a neurologist would know that I was in possession of the claimed invention, and described how to make and use the claimed invention, using the information provided in the specification.

7. I understand that the Office Action states that claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 (which have generally been rewritten as claims 106-136) are allegedly anticipated by the '855 patent. (Office Action, page 8) In addition, I also understand that the Office Action states that in the absence of evidence to the contrary, the neural stem cell compositions disclosed by Weiss *et al.* are indistinct from the cell compositions instantly claimed. (Office Action, page 9) I disagree with these statements, and I provide information below to show that the neural stem cell compositions disclosed by Weiss *et al.* are distinct from the cell compositions claimed in my application.

8. The information presented below (in paragraphs 10-15) demonstrates that neural precursor cells derived from neural tissue (embryonic, juvenile or adult) according to the Weiss method maintain their regional identity. The Office Action itself states that the '885 patent discloses mammalian neural stem cells, and that these cells can be derived from embryonic, juvenile, or adult mammalian neural tissue. (Office Action, page 9). Thus, neural precursor cells derived from a specific brain region are restricted. They will express specific markers for that region and will form the types of

neurons found in that region. Phenotypic restriction is a common feature of neural cells isolated from specific regions of the central nervous system and further propagated *in vitro*. This is in contrast to the neural precursor cells derived from embryonic stem cells of the present invention, which have the potential to generate all cell types of the nervous system.

9. My invention as set forth in the amended claims relates to a cell composition or neural spheres consisting essentially of “embryonic stem cell-derived” neural precursor cells or glial precursor cells (and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells as indicated in the claims) and additionally contain a step that requires “culturing embryonic stem cells to produce neural precursor cells.” In contrast, as stated in the Office Action, Weiss *et al.* disclose mammalian neural stem cells that can be derived from embryonic, juvenile, or adult mammalian neural tissue. (Office Action, page 9)

10. A neurologist would be aware that, unlike the neural precursor cells in the cell compositions claimed in my application, the neural precursor cells propagated according to Weiss *et al.* maintain their regional identity. Thus, neural precursor cells derived from a specific brain region are restricted. They will express specific markers for that region and will form the types of neurons found in that region. This fact is supported by Nakagawa *et al.*, (1996) *Development* 122:2449-2464 (attached hereto as Appendix B) and Ostenfeld *et al.*, (2002) *Devel. Brain Res.* 134:43-55 (attached hereto as Appendix C).

11. Nakagawa *et al.* provides “evidence that each neuroepithelial cell harbors some cell-autonomous mechanisms that direct the expression of a particular combination of region-specific genes, and that the same mechanisms also play an important role in regulating how the cell responds to inductive signals from the environment” (page 2450). This paper further states that results provided in the paper “raise the possibility that in neuroepithelial cells, certain cell-autonomous mechanisms

operate to maintain selective gene expression even after the cells are isolated from the original environment” (page 2454). The paper concludes that multipotential neural stem (MNS) cell lines “possess certain cell-autonomous mechanisms that maintain the expression of specific sets of region-specific genes *in vitro* even in the absence of environmental signals” (page 2457).

12. Nakagawa *et al.* established *in vitro* culture systems which enabled the authors to study how cell-autonomous mechanisms and environmental signals contribute to the regulation of region-specific genes in neuroepithelial cells. In their discussion, the authors state:

First, we showed that neuroepithelial cells from distinct regions of the brain express region-specific genes, including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Emx2* and *Dbx* at different levels. . . . These results support the notion that certain cell-autonomous mechanisms play an important role in maintaining the expression of particular sets of genes in neuroepithelial cells.

Next, we demonstrated that differential expression of region-specific transcription factors among different neuroepithelial cells can be reproduced in immortalized cell lines. MNS cell lines used in this study, which were established from E11.5 rat forebrain and midbrain (Nakafuku and Nakamura, 1995), shared properties of neural stem cells in that they expressed neuroepithelium-specific antigens and could generate neurons and glia under particular culture conditions . . . . These results strongly suggest that at least in some cases, expression of region-specific genes in undifferentiated neural stem cells is directly inherited to their neuronal (and possibly also glial) progeny.

. . . We consider that these results may suggest the intriguing possibility that each MNS cell line inherited a particular regional identity from the neuroepithelial cell from which it originated. . . .

Based on the above results, we can conclude that certain cell-autonomous mechanisms play important roles in

maintaining the expression of a specific set of region-specific genes. . . .

. . . Furthermore, we found that the combinations of the induced genes differed among different cell lines. These results strongly suggest that cell-intrinsic properties of neuroepithelial cells indeed define the responsiveness to environmental signals, as well as the repertoires of genes expressed in their absence. . . .

In summary, it is highly likely that both cell-autonomous mechanisms and environmental factors contribute cooperatively to the differential and regulated expression of the genes specifying the identities of neuroepithelial cells.

13. Therefore, this reference shows that for the cell lines used in this study (which were established from E11.5 rat forebrain and midbrain and shared properties of neural stem cells in that they expressed neuroepithelium-specific antigens and could generate neurons and glia under particular culture conditions) expression of region-specific genes in undifferentiated neural stem cells may be directly inherited by their neuronal (and possibly also glial) progeny.

14. In Ostenfeld *et al.*, neural precursor cells were isolated from various regions of the developing rat and human brain and grown in culture in aggregates termed neurospheres. The authors asked whether cells within human and rodent neurospheres are identical, or whether they have species specific characteristics or differences based on their region of origin. The data showed that rat and human neurospheres have unique characteristics with regard to growth and differentiation, and that the majority of precursor cells within neurospheres are regionally specified to generate set numbers of neurons (Abstract). Such regional differences included differences in expansion ratios (cell numbers), sphere size, and size of cell bodies (pages 45-47). The authors conclude that

The data presented here suggest that there are significant regional differences when neurospheres are derived from different areas of the developing rodent or

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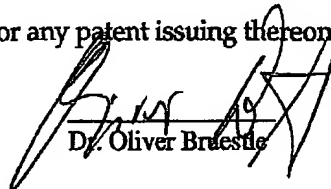
human brain. Thus, many of the cells dividing within neurospheres retain a memory of their origin. This may be a result of the different developmental stages the regions were at when cultured, or simply that each neurogenic zone of the developing brain has a specific type of cell which responds to EGF and FGF-2. Regardless of the mechanism underlying this phenomenon, it will be important for transplantation studies using cells derived from human neurospheres to establish their exact origin. (page 54)

Thus, at least some neural stem cells or neural precursor cells obtained from neural tissue will express region-specific genes, even when removed from the specific region of the brain and cultured *in vitro*. Furthermore, the neural precursor cells derived from a specific brain region will form the types of neurons found in this region.

15. In contrast, the cell compositions of the currently pending claims are derived from embryonic stem cells, not neural tissue. Accordingly, these cells will not exhibit the region-specific expression profile that would be seen in the cells of Weiss *et al.* They are not restricted with regard to the region-specific markers that they can express and the types of neural cells that they can become. Accordingly, it is my opinion as a skilled neurologist that the claimed cell compositions are different from those described in Weiss *et al.*

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

Date 4-24-06

  
Dr. Oliver Bruestle

## BIOGRAPHICAL SKETCH

|  |              |  |                    |
|--|--------------|--|--------------------|
| NAME, DOB<br>Prof. Oliver Brüstle, Oct 7, 1962 |              | POSITION TITLE<br>Scientific Director, LIFE & BRAIN GmbH<br><br>Director, Institute of Reconstructive Neurobiology, University of Bonn |                    |
| EDUCATION/TRAINING                             |              |  |                    |
| INSTITUTION AND LOCATION                       | DEGREE       | YEAR(s)  | FIELD OF STUDY     |
| University of Ulm, Medical School, Germany     | M.D.         | 1989   | Medicine           |
| University of Zurich, Switzerland              | Resident     | 1989-1991  | Neuropathology     |
| University of Erlangen-Nürnberg, Germany       | Resident     | 1991-1993  | Neurosurgery       |
| National Institutes of Health, Bethesda, MD    | Postdoc      | 1993-1997  | Stem cell research |
| University of Bonn, Medical Center, Germany    | Group Leader | 1997-2001  | Neuropathology     |

### A. Professional Positions

1989-1991 Resident; Institute of Neuropathology; University of Zurich, Switzerland; Head: Paul Kleihues, M.D.  
 1991-1993 Resident; Dept. of Neurosurgery; University of Erlangen-Nürnberg, Germany;  
 Head: Rudolf Fahlbusch, M.D.  
 1993-1997 Visiting Associate; Laboratory of Molecular Biology; National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD; Head: Ronald D. G. McKay, Ph.D.  
 1997-2001 Group Leader; Department of Neuropathology; University of Bonn Medical Center, Bonn, Germany  
 since 2002 Director, Institute of Reconstructive Neurobiology, University of Bonn Medical Center, Bonn  
 Co-Founder and Scientific Director, LIFE & BRAIN GmbH Bonn

### Honors:

1993-95 Fellowship award from the Deutsche Forschungsgemeinschaft  
 1995-97 Visiting Associate Fellowship from the NIH  
 2000 Bennisgen-Foerder-Preis  
 2004 X.O. Award (Financial Times Deutschland & Hennessy)

### B. Selected Publications

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## Roles of cell-autonomous mechanisms for differential expression of region-specific transcription factors in neuroepithelial cells

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### SUMMARY

Although a number of genes have been found to have restricted expression domains in the embryonic forebrain and midbrain, it remains largely unknown how the expression of these genes is regulated at the cellular level. In this study, we explored the mechanisms for the differential expression of region-specific transcription factors in neuroepithelial cells by using both primary and immortalized neuroepithelial cells from the rat brain at embryonic day 11.5. We found that differential expression patterns of *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2*, *Otx1* and *Dbx* observed in vivo were maintained even when the cells were isolated and cultured in vitro, free from environmental influences. Furthermore, in response to Sonic hedgehog, which is a major inductive signal from the environment for

regional specification, neuroepithelial cells that maintain distinct regional identities expressed different sets of ventral-specific genes including *Islet-1*, *Nkx-2.1* and *Nkx-2.2*. These results suggest that certain cell-autonomous mechanisms play important roles in regulating both environmental signal-dependent and -independent expression of region-specific genes. Thus, we propose that use of the in vitro culture systems we describe in this study facilitates the understanding of regulatory mechanisms of region-specific genes in neuroepithelial cells.

Key words: neural development, regional specification, neural stem cells, neuromere, homeobox gene, rat

### INTRODUCTION

The mammalian central nervous system is the center of higher cognitive functions. Its generation involves a number of events including neural induction, patterning of the neural plate, commitment and differentiation of neurons and glia, and establishment of neuronal and glial connections. These events collectively contribute to the generation of neural cell diversity. However, the molecular mechanisms controlling this complex process have just begun to be elucidated.

An important aspect of early development of the brain is the regional specification of neuroepithelial cells (Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Lumsden and Graham, 1995). For example, it is well established that the vertebrate hindbrain neuroepithelium is composed of discrete anatomical as well as functional units called rhombomeres, which serve as a framework for subsequent regional specification. In chick embryos, the cranial motor nerve nuclei are derived from adjoining pairs of rhombomeres (Lumsden, 1990), which indicates that neuroepithelial cells in each rhombomere are fated to give rise to particular types of neurons. Searches for

possible molecular correlates of the rhombomeres have succeeded in identifying many putative regulatory genes that show segment-specific expression domains. These include the *Antennapedia* class of homeobox-containing (*Hox*) genes (Wilkinson et al., 1989a) and the zinc-finger gene *Krox-20* (Wilkinson et al., 1989b). The boundaries of these expression domains have been shown to restrict the mixing of neuroepithelial cells (Birgbauer and Fraser, 1994) as well as the intercellular movement of small molecules via gap junctions (Martinez et al., 1992).

Although there have long been controversies over the early organization of more anterior parts of the brain, i.e. the forebrain and midbrain, the recent discoveries of genes that are expressed in a region-specific manner along with refined morphological studies have revealed that the vertebrate forebrain, like the hindbrain, can also be divided into discrete domains (Bulfone et al., 1993; Figdor and Stern, 1993; Puelles and Rubenstein, 1993; Macdonald et al., 1994). Bulfone et al. (1993) compared the expression domains of the *Dlx-1*, *Dlx-2*, *Gbx-2* and *Wnt-3* genes in the embryonic day (E) 12.5 mouse forebrain and concluded that the developing forebrain can be

divided into six transverse domains called prosomeres, and that each prosomere can be subdivided further into longitudinal domains. These proposed domain structures matched the neuromeric model of the forebrain organization based on morphological studies (Puelles et al., 1987). Analyses of expression domains of other genes from different families including the paired-domain-containing *Pax* family (Stoykova and Gruss, 1994) and the homeobox-containing *Otx*, *Emx* and *Nkx* families (Simeone et al., 1992a,b; Guazzi et al., 1990; Price et al., 1992; Shimamura et al., 1995) have provided further evidence for their hypothesis (see Boncinelli et al., 1993 and Rubenstein et al., 1994 for reviews). Studies of chick and zebrafish embryos revealed that such gene expression boundaries partially restrict cell mixing and are correlated with the positions of early generated neurons and their axonal tracts (Figdor and Stern, 1993; Wilson et al., 1993; Macdonald et al., 1994; Guthrie, 1995). Although the functions of these region-specific genes remain to be elucidated, some of them are expressed in specific populations of postmitotic neurons at later stages of development. Since many of these region-specific genes encode transcription factors, they are likely to serve as important regulators in the genetic network leading to the generation of particular neural cell types. Therefore, it is reasonable to expect that neuroepithelial cells expressing different combinations of region-specific transcription factors give rise to distinct types of neurons or glia.

In this respect, it is important to elucidate how the expression of region-specific genes is controlled in neuroepithelial cells. In particular, it has been a salient issue whether the patterns of gene expression and fate choices of neuroepithelial cells are defined by lineage-dependent cell-autonomous mechanisms or environment-dependent non-cell-autonomous regulation (Jessell and Dodd, 1992; Williams and Goldowitz, 1992; Lumsden et al., 1994; Johnson and Tabin, 1995; Simon et al., 1995). Several lines of evidence have suggested an important role for environmental regulation. Transplantation experiments of embryonic brain tissues and/or cells have revealed that neuroepithelial cells are plastic and can change their regional phenotypes dependent on their environments (Nakamura, 1988; Vicario-Abejón et al., 1995). In the chick spinal cord, the specification of dorsoventral identity of neuroepithelial cells is also altered by environmental signals: either removal or implantation of the notochord dramatically altered the dorsoventral expression patterns of *Pax-3* and *Pax-6* (Goulding et al., 1993). In this case, the secreted protein Sonic hedgehog (SHH), also termed Vhh-1 or Hhg-1, or its related proteins have been identified as signals from the notochord that control the restricted expression of these genes (Ekker et al., 1995; Liem et al., 1995). More recent studies have indicated that SHH is also involved in the induction of genes expressed in a subset of cells in the ventral forebrain including *Islet-1* (*Isl-1*), *Nkx-2.1*, *Nkx-2.2* and *Lim-1* (Ericson et al., 1995; Barth and Wilson, 1995; Lumsden and Graham, 1995). It has also been shown that members of the transforming growth factor- $\beta$  superfamily (BMP-4, BMP-7 and dorsalin-1) are involved in specification of the dorsoventral polarity in the spinal cord by regulating the expression of particular region-specific genes such as *Pax-3* and *Msx1* (Basler et al., 1993; Liem et al., 1995). These studies have emphasized the important role of the environment in defining the expression of region-specific genes.

In contrast, some previous studies have also demonstrated the importance of cell-autonomous mechanisms in maintaining regional identity of neuroepithelial cells. In the chick embryonic hindbrain, a fate-tracing study of single neuroepithelial cells (Lumsden et al., 1994) as well as transplantation studies of particular rhombomere segments (Guthrie et al., 1992; Simon et al., 1995) revealed a role for cell lineage in cell-type determination and *Hox* gene expression. Consistently, the existence of such mechanisms has also been implicated in the forebrain region concerning the expression of the limbic system-associated membrane protein (LAMP) and the PC3.1 antigen (latexin; Arimatsu et al., 1992; Ferri and Levitt, 1993). Furthermore, it has been shown very recently that the expression of the *Otx2*, *Emx2* and *Dlx-1* genes was maintained in neuroepithelial cells cultured in vitro (Robel et al., 1995). These studies have suggested that differential expression of particular antigens and genes in restricted regions of the rodent cerebral cortex was maintained in neuroepithelial cells by certain cell-autonomous mechanisms. However, these studies were carried out using mixtures of cells which are likely to be heterogeneous in terms of the expression of region-specific genes even if they were isolated from restricted areas or domains. Thus, it still remains to be clarified whether cell-autonomy is really operating at the single-cell level in maintaining particular regional identities.

To address these questions, we employed in vitro systems in which the involvement of cell-autonomous mechanisms and environmental signals can be independently manipulated and evaluated. In this study, we first examined the expression of region-specific transcription factors including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* in primary culture of neuroepithelial cells from three distinct regions of the developing (E11.5) brain; dorsal forebrain, ventral forebrain and caudal midbrain. Consistent with the results of previous in situ hybridization studies, each of the above genes was indeed differentially expressed in cell populations from distinct regions. We also found that such patterns were maintained when the cells were cultured for 5 days in vitro, suggesting that cell-autonomous mechanisms contribute to this phenomenon. To further explore this possibility, we utilized clonal neuroepithelial cell lines that we recently established from the E11.5 rat forebrain and midbrain (designated as MNS cell lines; Nakafuku and Nakamura, 1995). All of the five cell lines which we characterized shared properties of undifferentiated neuroepithelial cells and could give rise to neurons and glia under particular culture conditions. We demonstrate that each of these cell lines maintained expression of a specific combination of region-specific transcription factors not only during their clonal expansion in vitro, but also when the cells were induced to differentiate. Furthermore, we show that SHH induced different sets of ventral-specific genes in primary culture of distinct regions of the embryonic neuroepithelium as well as in distinct MNS cell lines. These results provide evidence that each neuroepithelial cell harbors some cell-autonomous mechanisms that direct the expression of a particular combination of region-specific genes, and that the same mechanisms also play an important role in regulating how the cell responds to inductive signals from the environment.

## MATERIALS AND METHODS

### Primary culture of neuroepithelial cells

Neuroepithelia of the forebrain and midbrain were dissected out from E11.5 Sprague-Dawley rats as described previously (Nakafuku and Nakamura, 1995). The day on which the copulatory plug was found was considered as E0.5. Isolation of neuroepithelial cells from specified areas was carried out as follows; the prospective cerebral cortex was cut out from the embryos and used as a dorsal forebrain preparation. From the remaining embryonic head, the ventral forebrain and caudal midbrain regions were dissected out, and surrounding mesenchymal tissues were removed from the primary neuroepithelia. The uppermost position of the optic vesicle and caudal edge of the forebrain vesicle were used as landmarks of the dorsal and caudal margins, respectively, of the ventral forebrain preparations. The rostral margin of the caudal midbrain preparations was defined as half way between the edge of the forebrain vesicle and the rhombencephalic fissure. We noticed that by means of the above method, it was difficult to completely eliminate the most anterior portion of the neural tube, the prospective septal region, from the dorsal forebrain preparation, since no morphological landmark for that region was apparent by inspection at this stage (see Results for details). To further eliminate contaminating non-neural tissues, isolated tissue pieces were incubated at 4°C for 20 minutes in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium (DF; Sanko Junyaku) containing 0.05% (w/v) trypsin and 3 mM sodium ethylenediamine tetraacetic acid (EDTA) as described previously (Murphy et al., 1990). Subsequently, the samples were collected into test tubes and washed three times with DF medium. The preparations were then treated with DF medium containing 0.1% trypsin, 0.001% DNase I (Sigma) and 3 mM EDTA at 37°C for 15 minutes, and single-cell suspensions were made by repeated gentle pipetting. The dissociated cells were divided into two and the first half was directly collected (day 0 preparation). The other half was plated onto poly-D-lysine (10 µg/ml)-coated dishes at densities between 2 to 5 × 10<sup>5</sup> cells per cm<sup>2</sup> in a standard culture medium consisting of 10% fetal bovine serum (FBS; Sanko Junyaku), 5% horse serum (HS; Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin in DF medium, and was cultured in vitro for 5 days (day 5 preparation). Immunocytochemical studies showed that more than 98% of the isolated cells were positively stained by anti-nestin and the RC1 antibodies, indicating that the preparations were essentially free of non-neural cells. After 5 days in vitro, total cell numbers increased 2- to 3-fold, and the percentage of nestin-positive cells decreased to 65-70%, whereas MAP2-positive neurons and GFAP-positive astrocytes emerged at percentages of 10-20% and 1-5% of the total cells, respectively.

### Establishment and maintenance of neuroepithelial cell lines

The neural stem cell lines used in this study, herein designated as MNS (multipotential neural stem) cell lines, were established and maintained as described previously (Nakafuku and Nakamura, 1995). Primary cultured neuroepithelial cells prepared from E11.5 rat forebrain and midbrain were immortalized by infection with the recombinant retrovirus from the -2myc cell-conditioned medium (Eilers et al., 1989). The retrovirus carries the *c-myc* gene whereby *c-myc* is fused to the ligand-binding domain of the estrogen receptor. With this system it is possible to conditionally activate the *c-myc* protein by adding estrogen (e.g. -E2) to the culture medium (Eilers et al., 1989). Since the retrovirus also carries the *neo<sup>r</sup>* gene, infected cells were selected with G418. The clonality of the characterized cell lines was confirmed by Southern blot analysis of the provirus integration sites (data not shown). Each cell line was maintained in monolayer culture in the standard medium described above ('monolayer culture'). The medium was changed every 3 days. The

detailed procedures to induce differentiation of the cells were described previously (Nakafuku and Nakamura, 1995). Briefly, cells were first allowed to form aggregates in suspension for 3 days in standard medium containing 20 ng/ml bFGF and 1 µM -E2 ('aggregation culture'). Culture dishes were coated with poly [2-hydroxyethyl methacrylate] (poly HEMA; Sigma) to avoid cell attachment. Cell aggregates were then re-seeded onto poly-D-lysine (100 µg/ml)-coated dishes and were cultured in differentiation medium (standard medium without HS) for 3 or 4 days ('differentiation culture'). The cells cultured under each of the three different conditions were subjected to immunocytochemical studies and RNA preparation.

### Expression of SHH and treatment of primary culture and MNS cell lines

CV1 cells stably expressing SHH (CV1SHH) were produced by transfection with a plasmid harboring the full-length chicken SHH cDNA (Ogura et al., 1996). The expression of the N-terminal cleavage product of SHH in CV1SHH cells and in their conditioned media was analyzed using the affinity-purified rabbit anti-SHH antibody (Ab 80) as described by Bumcrot et al. (1995), (a gift from Dr A. McMahon), in which cell pellets were directly lysed in Laemmli's sample buffer, whereas proteins in conditioned medium were concentrated 10-fold by precipitation with 10% trichloroacetic acid. In the case of primary culture of neuroepithelial cells, media conditioned for 48 hours by confluent monolayer of CV1SHH cells and the parental CV1 cells were used for the treatment with SHH and its control, respectively. Preparations of the dorsal and ventral forebrain were isolated and used exactly as described above. For the caudal midbrain, however, its ventral two-third was removed, and the remaining dorsal portion was used as a preparation of the dorsal midbrain. MNS cell lines were treated with SHH by culturing them in contact with a confluent monolayer of CV1SHH cells for 3 days. Control cells were cocultured with the parental CV1 cells in stead of CV1SHH cells. During this 3 day-culture period, no apparent differences in either growth property or viability were observed between the cells in contact with CV1 and those incubated with CV1SHH cells.

### Immunostaining

The antibodies used for immunocytochemical studies were described previously in detail (Nakafuku and Nakamura, 1995) with the following exceptions: anti-Pax-6 mouse monoclonal antibody (mmAb; a generous gift from Drs H. Fujisawa and A. Kawakami, Nagoya University; Kawakami et al., unpublished data); anti-MAP2 polyclonal antibody (diluted 1:500; provided by Dr Y. Ihara, University of Tokyo); and the R24 anti-GD3 ganglioside mmAb (LeVine and Goldman, 1988, undiluted conditioned medium of hybridoma, from the ATCC Hybridoma Bank). Indirect immunocytochemical detection of various antigens was performed as previously described (Nakafuku and Nakamura, 1995). A2B5 and R24 antibodies specifically labeled cells in the oligodendrocyte lineage, but not MAP2-positive neurons or GFAP-positive astrocytes under our experimental conditions. The immunoreactive cells were visualized using fluorescein isothiocyanate (FITC)- or texas red (TR)-conjugated species-specific secondary antibodies (diluted 1:50-100; Cappel or Amersham).

### RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from cells or tissues using the acid guanidinium-phenol-chloroform (AGPC) extraction method as described previously (Chomczynski and Sacchi, 1987). RNAs of primary neuroepithelial cells were prepared from day 0 and day 5 cultures (See above for detailed culture protocol). RNAs from MNS cell lines were prepared from three different types of culture: monolayer culture, aggregation culture and differentiation culture (see above). RNA was also prepared from tissues of E11.5 and E15.5 rat forebrain/midbrain

and E11.5 whole embryos deprived of forebrain/midbrain for use as control.

Relative expression levels of various genes in tissue and cultured cell samples were compared by quantitative RT-PCR analysis. cDNAs were synthesized from total RNA by MuMLV reverse transcriptase (Superscript II; Gibco) at 50°C for 1 hour. cDNA derived from 40 ng of total RNA was amplified in a 100 µl PCR reaction containing 5 units of Taq DNA polymerase (Boehringer Mannheim), 1-PCR buffer, 0.4 mM of each dNTP and 100 pmols of each primer (see Table 1) in a thermal cycler (Perkin Elmer, denaturation for 1 minute at 94°C, annealing for 1 minute at 56°C and extension for 2 minutes at 72°C). For *Wnt-3* and *En-1* transcripts, annealing was carried out at 62°C and 58°C, respectively.

Oligonucleotides used to amplify the cDNAs are listed in Table 1. The identities of the PCR products were confirmed by sequencing the subcloned fragments using an automated sequencer (A.L.F. Sequencer II, Pharmacia and ABI 373A DNA Sequencer, Applied Biosystems). For the *Otx* genes, we used degenerate primers that can detect both *Otx1* and *Otx2*. At the time of this study, the rat sequences were not available for the *Pax-3*, *Pax-5*, *Dlx-1*, *Otx1*, *Emx2*, *Dbx*, *Wnt-3*, *En-1*, *Hox-B1*, *Hox-B3*, or *Nkx-2.2* genes. For these genes, we used the mouse sequences for primer design and compared the similarity of the obtained PCR products with the mouse sequences. More than

97% identity in nucleotide sequence was observed in each clone, which gave sufficient information to distinguish different members of each family. Therefore, we concluded that the obtained cDNA clones were the cognate rat counterparts of the mouse cDNAs.

Relative quantification of gene expression by use of RT-PCR was carried out as follows. During each set of PCR reactions, 8 µl aliquots were collected from the reaction mixtures every 2-3 cycles, and 4 µl of the samples were electrophoresed in 1% agarose or 6% polyacrylamide gels. The gels were then stained with 0.01% SYBR Green I (Amersham) for 45 minutes and fluorescence intensity was measured using FluorImager SI (Molecular Dynamics). For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification. In control experiments, we confirmed that relative levels of cDNA for a given gene present in different samples could be quantified within the range 20 pg to 10 ng by means of this method (Schneeberger et al., 1995 and Y. N., unpublished data). In all experiments, amplification of  $\alpha$ -actin cDNA was carried out alongside, and was used to normalize different cDNA samples. We repeated the above experiments three to five times for each gene using two to five independent preparations of tissue or cell samples, and the normalized mean values ( $\pm$ s.d.) are shown in figures and tables. Cycle numbers used for quantification are also shown in the legends to Figs 2, 4, 7 and 9, and Table 2. To

Table 1. PCR primers used in this study

| Gene                            | Sequence   | Reference              |
|---------------------------------|--|------------------------|
| <i><math>\beta</math>-actin</i> | Sense: 5'-TGC CCA TCT ATG AGG GTT ACG-3'<br>Antisense: 5'-TAG AAG CAT TTG CGG TGC ACG-3'                           | Nudel et al. 1983      |
| <i>MAP2</i>                     | Sense: 5'-GAA GGA AAG GCA CCA CAC TG-3'<br>Antisense: 5'-CGT GGC GAT GGT GGT GGG-3'                                | Kindler et al. 1990    |
| <i>GFAP</i>                     | Sense: 5'-CAA GCC AGA CCT CAC AGC G-3'<br>Antisense: 5'-GGT GTC CAG GCT GGT TTC TC-3'                              | Lewis et al. 1984      |
| <i>CNP-II</i>                   | Sense: 5'-CCG GAG ACA TAG TGC CCG CA-3'<br>Antisense: 5'-AAA GCT GGT CCA GCC GTT CC-3'                             | Gravel et al. 1994     |
| <i>Pax-3</i>                    | Sense: 5'-GCT GTC TGT GAT CGG AAC ACT-3'<br>Antisense: 5'-CTC CAG CTT GTT TCC TCC ATC-3'                           | Goulding et al. 1991   |
| <i>Pax-5</i>                    | Sense: 5'-GAG CGG GTG TGT GAC AAT GAC-3'<br>Antisense: 5'-CGA GGC CAT GGC TGA ATA CTC-3'                           | Adams et al. 1992      |
| <i>Pax-6</i>                    | Sense: 5'-AGT CAC AOC GGA GTG AAT CAG C-3'<br>Antisense: 5'-AGC CAG GTT GCG AAG AAC TCT G-3'                       | Walther and Gruss 1991 |
| <i>Dlx-1</i>                    | Sense: 5'-CAA GGC GGG GCA GCT CTG-3'<br>Antisense: 5'-GGG AGA CGG GCA GGA AGC-3'                                   | Price et al. 1991      |
| <i>Dlx-2</i>                    | Sense: 5'-AGG ATG ACT GGA GTC TTT GAC-3'<br>Antisense: 5'-TCG GAT TTC AGG CTC AAG GTC-3'                           | Porteus et al. 1992    |
| <i>Otx</i>                      | Sense: 5'-TAT/C CCI GCI ACI CCI A/CGA/G AAA/G CA-3'<br>Antisense: 5'-ACI AA/GT/C TGT/C TGI CT/GA/G CAT/C TTI GC-3' | Simeone et al. 1992a   |
| <i>Emx2</i>                     | Sense: 5'-GTC CCA GCT TTT AAG GCT AGA-3'<br>Antisense: 5'-CTT TTG CCT TTT GAA TTT CGT TC-3'                        | Simeone et al. 1992b   |
| <i>Dbx</i>                      | Sense: 5'-GCA GA/CG A/GAA A/GGC/G C/GCT GGA GAA-3'<br>Antisense: 5'-TA/GG AA/GT T/GCC GCC AC/TT TCA TC/GC-3'       | Lu et al. 1992         |
| <i>Wnt-3</i>                    | Sense: 5'-GAA GGC TGG AAG TGG GGC GGC-3'<br>Antisense: 5'-ACG CAA TGG CAT TTC TCC TTC CG-3'                        | Roelink et al. 1990    |
| <i>En-1</i>                     | Sense: 5'-GAC AGT GGC GGT GGT AGT G-3'<br>Antisense: 5'-GAG GAG CCT GGA GGT GGC-3'                                 | Joyner et al. 1987     |
| <i>Hox-B1</i>                   | Sense: 5'-CCG GAC CTT CGA CTG GAT G-3'<br>Antisense: 5'-GGT CAG AGG CAT CTC CAG C-3'                               | Wilkinson et al. 1989a |
| <i>Hox-B3</i>                   | Sense: 5'-GTC GAC GCA AAC TGC CAA GC-3'<br>Antisense: 5'-GGG TCA TGG AGT GTA AGG CG-3'                             | Wilkinson et al. 1989a |
| <i>Nkx2.1</i>                   | Sense: 5'-GGC CAT CTC TGT GGG CAG C-3'<br>Antisense: 5'-CTC AGG CGC GTC CCA CAT C-3'                               | Guazzi et al. 1990     |
| <i>Nkx2.2</i>                   | Sense: 5'-GGG GGA NCGC AGG CAA GAA G-3'<br>Antisense: 5'-TGT AGG CCG AAA AGG GGA TG-3'                             | Price et al. 1992      |
| <i>Isl-1</i>                    | Sense: 5'-GCA GCA TAG GCT TCA GCA AG-3'<br>Antisense: 5'-GTA GCA GGT CCG CAA GGT G-3'                              | Karlsson et al. 1990   |

visualize the difference in expression levels among different samples, direct printouts from the fluorescence image analyzer are shown in Figs 4A, 5 and 9C. In these cases,  $\beta$ -actin-normalized cDNA templates were amplified at fixed cycle numbers. For clearer visualization of PCR products, cycle numbers in some cases were 2-3 times larger than those used for quantification, but they were still within the range of exponential amplification (see figure legends for details).

## RESULTS

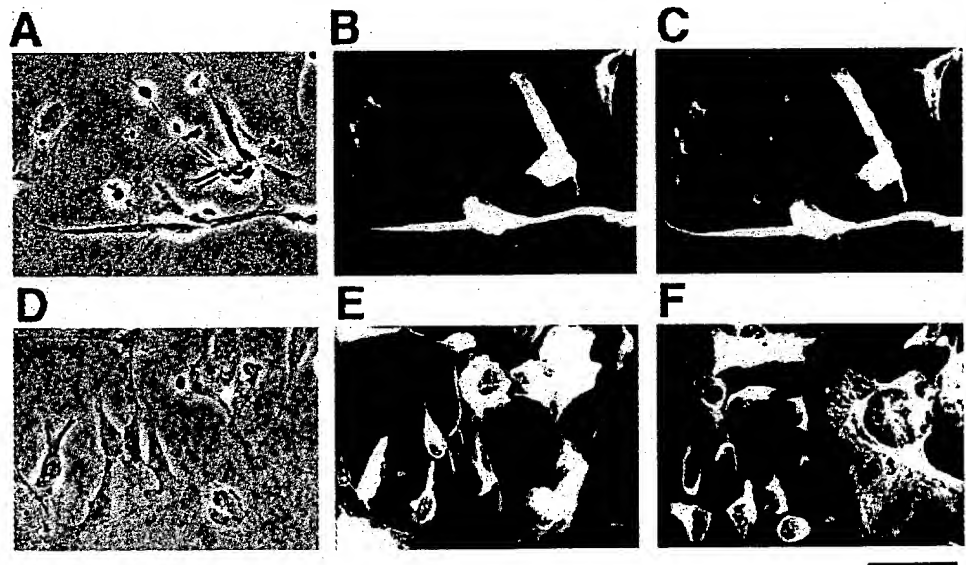
### Expression of region-specific genes in neuroepithelial cells in primary culture

To explore the mechanisms that control differential expression of region-specific genes among discrete domains of the forebrain and midbrain, we first examined the expression of a set of transcription factors in neuroepithelial cells cultured in vitro. Neuroepithelia of three distinct regions, including the dorsal forebrain, ventral forebrain and caudal midbrain were dissected out from embryonic rat brain at E11.5 (which roughly corresponds to E9.5-10.5 in mice). Immunocytochemical studies showed that more than 98% of the cells in all the preparations expressed nestin (Lendahl et al., 1990) and RC1 antigen (Edwards et al., 1990), both of which are specific markers for undifferentiated neuroepithelial cells (Fig. 1A-C). From these tissue samples, we isolated RNAs either immediately after dissociation (day of in vitro culture [DIV] 0) or after 5 days in culture (DIV 5), and compared the expression levels of *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes by quantitative RT-PCR analysis (Fig. 2).

We first focused on the three members of the *Pax* gene family, *Pax-3*, *Pax-5* and *Pax-6*, which encode transcription factors related to the *Drosophila* protein paired (Stuart et al., 1993). In situ hybridization studies have shown that *Pax-3*, *Pax-5* and *Pax-6* expressions are first observed by E8.5 in mice along the entire anterior-posterior axis of the neural tube. At later stages, however, their expression domains become restricted to particular areas within the forebrain and midbrain. The rostral limits of *Pax-3* (Goulding et al., 1991) and *Pax-5* (Asano and Gruss, 1992) expression domains retract caudally to dorsal and ventral midbrain, respectively, whereas strong expression of *Pax-6* remains in the dorsal telencephalon at E13.5. In addition, other areas such as ventral thalamus, epithalamus and the ventral midbrain also show significant *Pax-6* expression (Walther and Gruss, 1991). Consistent with these in

vivo data, the levels of *Pax-3* and *Pax-5* mRNA were three to twenty times higher in DIV 0 cells of the caudal midbrain preparations than those in the ventral and dorsal forebrain, whereas *Pax-6* was expressed at comparable levels in all of these three regions (Fig. 2).

Next, we examined the expression of three classes of homeobox genes including the *Dlx*, *Emx* and *Dbx* genes, which are vertebrate homologues of the *Drosophila* *Distal-less* (Boncinelli, 1994; Price, 1993), *empty spiracles*, (Boncinelli et al., 1993) and *H2*, respectively. In the developing mouse brain, *Dlx-1* and *Dlx-2* share most of their expression domains, which reside predominantly in the forebrain, particularly in ventral regions (Bulfone et al., 1993). At E9.5 to 9.75 in mouse embryos, strong expression of *Emx2* is restricted both in the dorsal telencephalon and ventral diencephalon (Simeone et al., 1992b; Shimamura et al., 1995). As development proceeds to E12.5, however, it becomes detectable also in other regions such as the dorsal midbrain (Simeone et al., 1992b). Strong expression of *Dbx* is observed in the midbrain and in some ventral regions of the forebrain, but is scarce in the dorsal forebrain (Lu et al., 1992; N. Takahashi, personal communication). The results of RT-PCR analysis matched the above observations; the levels of *Dlx-1*, *Dlx-2* and *Emx2* expression in the DIV 0 cells from the ventral forebrain were higher (34, 6 and 4 times, respectively) than those from the midbrain, whereas the *Dbx* expression was eight times stronger in the midbrain than in the dorsal forebrain. Comparison of the expression of the above genes between the dorsal and ventral forebrain regions, however, has raised several issues that should be addressed. The expression levels of *Emx-2* in the



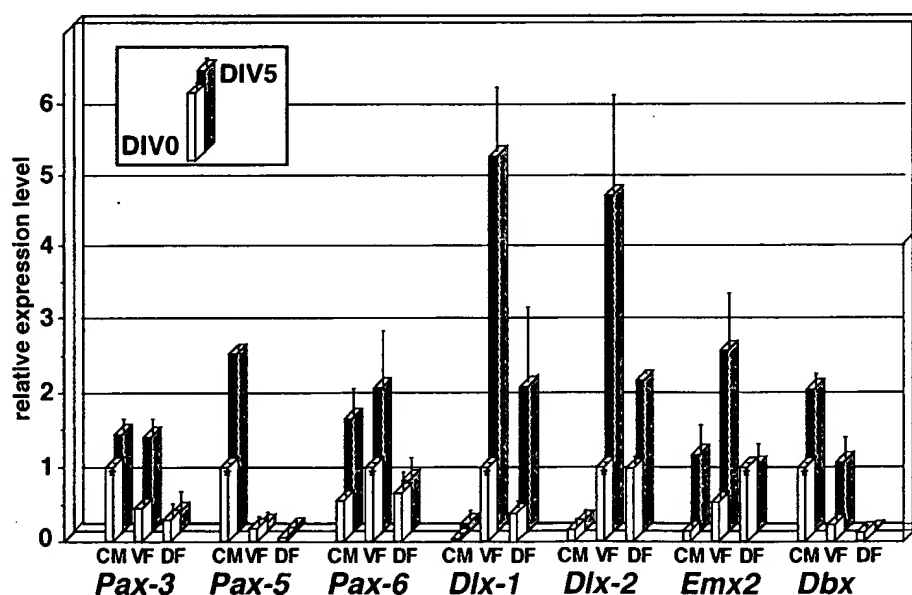
**Fig. 1.** Indirect immunocytochemical analysis for neuroepithelial cell antigens in primary neuroepithelial cells and MNS cell lines. Primary neuroepithelial cells from E11.5 rat forebrain and midbrain (A-C) and MNS-8 cells (D-F) were stained with antibodies that detect neuroepithelium-expressed antigens. (A) Phase contrast micrograph of primary neuroepithelial cells. The same population of cells was immunoreactive with anti-nestin (B) and the RC1 (C) antibodies. A-C show the same field. In our preparations of neuroepithelia from separate regions, more than 98% of the cells were always nestin/RC1-double positive at DIV 0 (data not shown). (D) Phase contrast micrographs of MNS-8 cells. Fluorescence micrographs showing the expression of nestin (E) and RC1 (F). D and E show the same field. Scale bar, 100  $\mu$ m.

dorsal forebrain was about twice as high as that in the ventral forebrain, which is generally consistent with the results of previous *in situ* hybridization studies (Simeone et al., 1992b; Shimamura et al., 1995). In contrast, only three times higher expression of *Dlx-1* and *Dbx* was observed in the ventral than that in the dorsal forebrain, which was smaller than expected from previous studies (Bulfone et al., 1993; Lu et al., 1992). In addition, although the expression of *Dlx-2* is reported to be predominant in the ventral rather than in the dorsal forebrain, it was expressed at similar levels in our preparations of the two regions. One possible reason for these results is that a part of the prospective septal region, which also abundantly expresses *Dlx-1*, *Dlx-2* and *Dbx*, was included in our preparation of the dorsal forebrain. Yet, the overall profiles (also see below) suggest that the results shown here reflect some parts of the differential gene expression patterns between the dorsal and ventral sides of the forebrain.

Next, to test whether the selective expression profiles of the above genes are maintained upon proliferation and differentiation of neuroepithelial cells, we cultured these cells for 5 days *in vitro*. During this culture period, neuroepithelial cells underwent significant proliferation as well as differentiation into neurons and glia (data not shown), and the cells from each of the three regions maintained gene expression profiles typical of region-specific genes. For example, *Pax-5* expression remained very low in cells derived from the ventral and dorsal forebrain, whereas expression of *Dlx-1* and *Dlx-2* genes remained high in the ventral forebrain. Likewise, both low levels of *Dlx-1* and *Dlx-2* and high levels of *Pax-3*, *Pax-5* and *Dbx* were maintained in cells from the caudal midbrain after *in vitro* culture. We noticed several exceptions to this general feature: firstly, *Pax-3* was expressed at a significant level in the ventral forebrain, and was even upregulated after 5 days of culture; secondly, *Emx2* and *Dbx* showed marked upregulation in the DIV 5 preparations of caudal midbrain and ventral forebrain, respectively. These observations can be explained by the shift in their expression domains during development. As described above, *Pax-3* expression is initially observed along the entire anterior-posterior axis of the neural tube, and its retraction in the forebrain region occurs between E11–12 in mice, which corresponds to E13–14 in rats (Goulding et al., 1991). Thus, it appears that our DIV 0 preparation of the ventral forebrain at

E11.5 still contained a significant population of *Pax-3*-positive cells. The upregulation of *Emx2* and *Dbx* in DIV 5 cells is also consistent with the extension of their strong expression domains into the midbrain and ventral forebrain at E12.5 in mice as previously described in detail (Simeone et al., 1992b; Lu et al., 1992). In particular, the change in *Emx2* expression from a dorsally enriched to a ventrally enriched pattern after 5 days in culture is consistent with the previous observation that the expression of *Emx2* declines in the dorsal forebrain accompanying the differentiation of cortical neurons, whereas it persists in the ventral regions including hypothalamus even at E17.5 (Simeone et al., 1992b). Thus, we can consider that the expression patterns of the above genes in neuroepithelial cells cultured *in vitro* reflect, at least in part, their spatial and temporal dynamics observed *in vivo*.

Taken together, these results raise the possibility that in neuroepithelial cells, certain cell-autonomous mechanisms operate to maintain selective gene expression even after the cells are isolated from the original environment. It is notable that Robel et al. (1995) reported very recently that the expression of *Otx2*, *Emx1* and *Dlx-1* was maintained in telencephalon-derived neuroepithelial cells *in vitro*, which is consistent with our findings. However, primary cultures inevitably contain heterogeneous



**Fig. 2.** Analysis of the expression of various region-specific genes in primary cultures of neuroepithelial cells. Quantitative RT-PCR analyses were carried out to examine the expression of the *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* genes in the primary cultures of neuroepithelial cells from different regions of E11.5 rat brain. Neuroepithelial cells were prepared from the caudal midbrain (CM), ventral forebrain (VF), and dorsal forebrain (DF) as described in Materials and Methods. Total RNA from these cells immediately after dissection (DIV 0; open bars) or after 5 days *in vitro* (DIV 5; hatched bars) was used for RT-PCR analyses. PCR products were electrophoresed, stained by SYBR Green I, and quantified by measuring the fluorescence intensity. For each primer pair, amplified PCR products among different samples were quantified at various cycles within the range of exponential amplification, and further normalized with the levels of  $\beta$ -actin transcript. For each gene, relative expression levels are shown as mean  $\pm$  s.d. of three independent reactions, where the highest expression level among the DIV 0 samples from the three brain regions (marked with asterisks) was designated as 1. Essentially identical results were obtained for five independent preparations. Data shown are from one representative preparation. The cycle numbers of PCR reactions used were: 35 for *Pax-3*; 35 for *Pax-5*; 35 for *Pax-6*; 29 for *Dlx-1*; 30 for *Dlx-2*; 35 for *Emx2*; 35 for *Dbx*; and 20 for  $\beta$ -actin.

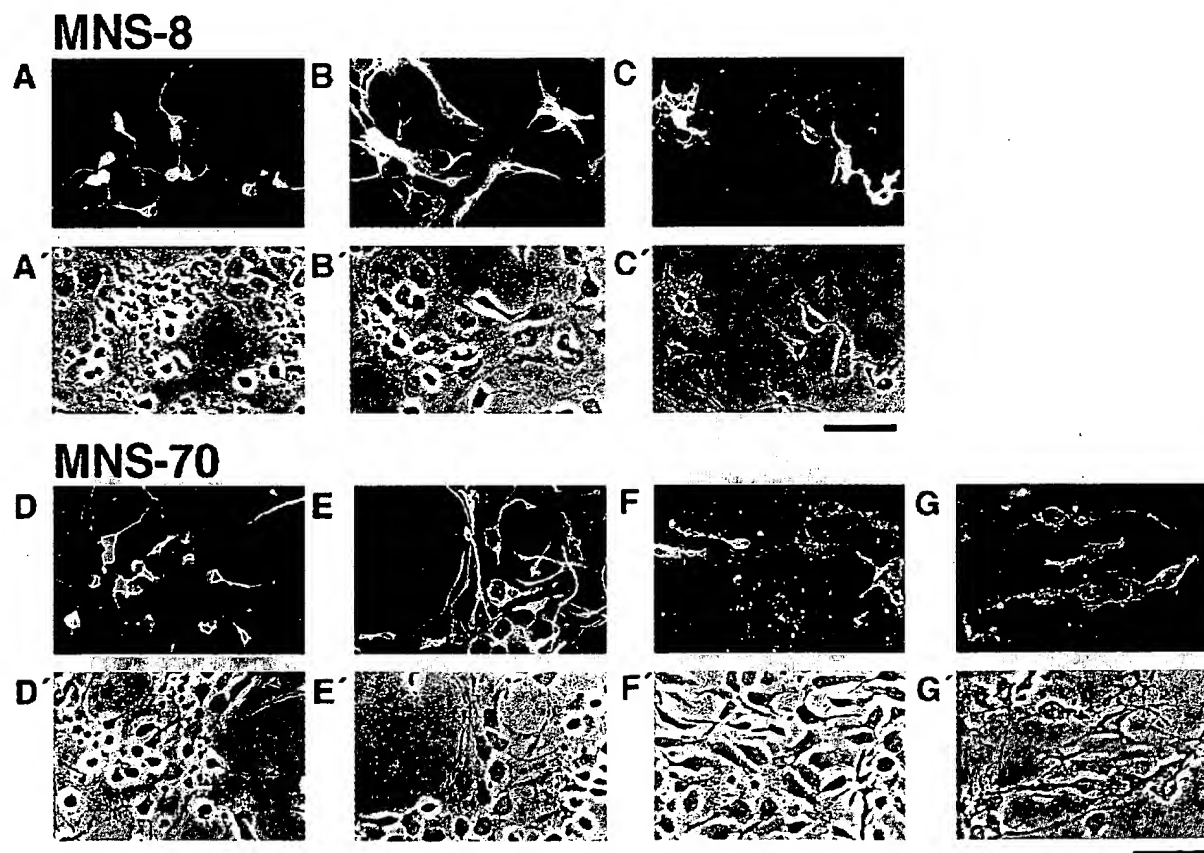
cell populations expressing different combinations of region-specific genes. Therefore, it is still possible that the maintenance of expression of particular sets of genes is due to non-cell autonomous interactions among cells with different regional identities. Furthermore, we could not examine whether a given single cell in culture continued to express the same sets of genes when it underwent proliferation and/or differentiation. To clarify these points more definitively, it is necessary to analyze clonal and homogeneous cell populations whose proliferation and differentiation can be manipulated *in vitro*. Therefore, we have established several clonally distinct neuroepithelial cell lines from the E11.5 rat forebrain and midbrain.

### MNS cell lines exhibit the properties of multipotential neural stem cells

Detailed characteristics of one of the cell lines used in this study (designated as MNS cell lines) were described previously (Nakafuku and Nakamura, 1995). Here, we show that the established MNS cell lines represent the properties of cultured neuroepithelial cells. We first examined the expression of nestin and the RC1 antigen. MNS-8 cells, like MNS-57 cells that were characterized previously (Nakafuku and Nakamura, 1995), expressed both antigens (Fig. 1D-F). Other clonally

distinct cell lines, including MNS-70, -71 and -92, showed similar antigenic phenotypes (data not shown). All these cell lines maintained in monolayer culture continued to express these antigens, indicating that they represent undifferentiated neuroepithelial cells.

We also examined the differentiation potentials of the MNS cell lines. By using the three-step culture protocol (monolayer, aggregation and differentiation culture; described in detail in Materials and Methods), we induced differentiation of the cells, and identified neurons, astrocytes and oligodendrocytes by specific antibodies against microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP) and galactocerebroside (GC), respectively. MNS-8 cells, like MNS-57 cells as described previously (Nakafuku and Nakamura, 1995), gave rise to these three cell lineages, whereas MNS-70, -71 and -92 cells generated only MAP2-positive neurons and GFAP-positive astrocytes, but not GC-positive oligodendrocytes under the conditions used in this study (Fig. 3 for MNS-8 and -70; data not shown for the other cells). However, the A2B5 (Gard and Pfeiffer, 1990) and R24 (LeVine and Goldman, 1988) antibodies, which recognize Gq and GD3 gangliosides on the cell surface, respectively, specifically labeled oligodendrocyte precursor cells with typical unipolar or bipolar morphologies in the differentiated cultures of all the cell lines.



**Fig. 3.** Generation of both neurons and glia in cultures of MNS cells after induction of differentiation. The cells in 'differentiation culture' (for details, see Materials and Methods) of MNS-8 (A-C) and MNS-70 cells (D-G) were subjected to immunocytochemical analyses using antibodies against various neuron- and glia-specific antigens. Immunofluorescent (A-G) and phase-contrast (A'-G') images of the same fields are shown side by side. The primary antibodies used were; A and D, anti-MAP2 antibody; B and E, anti-GFAP antibody; C, anti-GC antibody; F, A2B5 antibody; and G, R24 antibody. Scale bar, 50  $\mu$ m.

**Table 2. Expression patterns of various region-specific genes in the five MNS cell lines**

|              | Tissue    |                 |            | MNS cell lines |           |           |           |            |  |
|--------------|-----------|-----------------|------------|----------------|-----------|-----------|-----------|------------|--|
|              | E11.5     |                 | E16        | 8              | 57        | 70        | 71        | 92         |  |
|              | F/M brain | High expression | F/M brain  |                |           |           |           |            |  |
| <i>Pax-3</i> | 1.00      | 7.91±0.54 (CM)  | 0.88±0.14  | 0.03±0.02      | 0.03±0.03 | 0.03±0.02 | 3.64±0.59 | <0.01      |  |
| <i>Pax-5</i> | 1.00      | 5.93±1.00 (CM)  | 1.18±0.21  | <0.01          | 0.43±0.07 | 0.01±0.01 | 0.01±0.01 | 0.01±0.01  |  |
| <i>Pax-6</i> | 1.00      | 7.07±1.33 (VF)  | 2.32±0.73  | 0.52±0.08      | 2.37±0.44 | 2.40±0.39 | 0.28±0.11 | 0.04±0.02  |  |
| <i>Dlx-1</i> | 1.00      | 17.11±6.53 (VF) | 12.88±2.93 | 0.18±0.03      | 3.65±1.02 | 0.82±0.19 | 8.88±1.96 | 0.73±0.025 |  |
| <i>Dlx-2</i> | 1.00      | 7.65±1.38 (VF)  | 3.23±0.65  | 0.07±0.04      | 1.96±1.13 | 1.46±0.30 | 3.59±1.32 | 0.01±0.00  |  |
| <i>Otx1</i>  | 1.00      | ND              | 0.85±0.24  | 0.40±0.10      | 2.97±0.69 | 0.20±0.10 | 0.76±0.20 | 0.10±0.02  |  |
| <i>Emx2</i>  | 1.00      | 14.4±1.80 (DF)  | 2.45±0.58  | 0.41±0.10      | 1.87±0.35 | 0.58±0.24 | 0.13±0.03 | 1.22±0.32  |  |
| <i>Dbx</i>   | 1.00      | 18.23±2.63 (CM) | 1.29±0.46  | 0.02±0.02      | 0.64±0.36 | 8.94±4.46 | 0.66±0.50 | 0.05±0.02  |  |

In all cases,  $\beta$ -actin was used as an internal control and the expression level of each gene in the control E11.5 forebrain and midbrain tissue sample (F/M brain) was designated as 1.00. All the data shown are mean and s.d. values of three to five independent experiments. The highest level of each gene among the three distinct regions examined in Fig. 2 (indicated in parentheses) are also shown (High Expression). The cycle numbers of PCR used for quantification were: 31 for *Pax-3*; 35 for *Pax-5*; 35 for *Pax-6*; 30 for *Dlx-1*; 32 for *Dlx-2*; 33 for *Otx1*; 32 for *Emx2*; 35 for *Dbx*; and 21 for  $\beta$ -actin. DF, dorsal forebrain; VF, ventral forebrain; CM, caudal midbrain; ND, not determined.

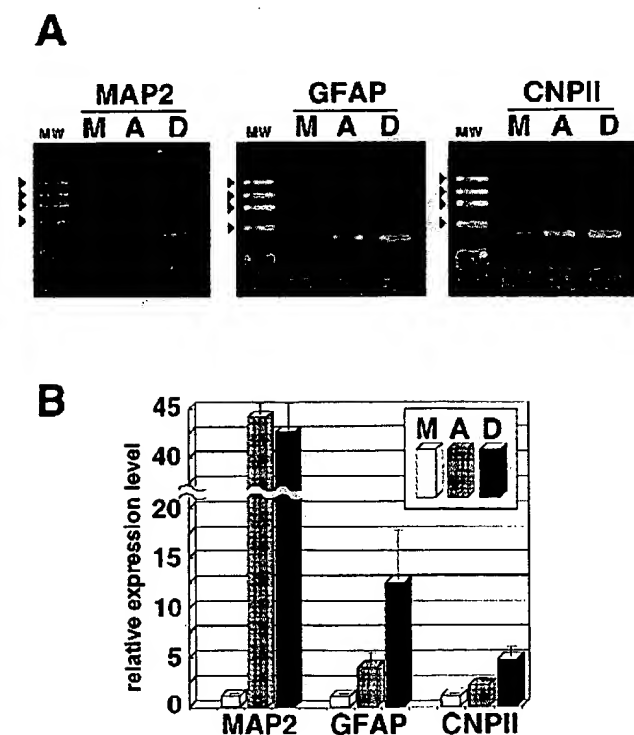
In addition to these immunological studies, RT-PCR analyses also demonstrated the expression of lineage-specific genes upon differentiation of the MNS cell lines. Representative results for MNS-8 cells are shown in Fig. 4A and B, in which levels of mRNAs for MAP2, GFAP and 2-3-cyclic nucleotide 3-phosphodiesterase isoform II (CNPII, a marker for oligodendrocytes; Scherer et al., 1994) increased upon induction of differentiation. Based on the above results, we concluded that the MNS cell lines share properties of multipotential neural stem cells present in original preparations of E11.5 forebrain/midbrain neuroepithelium.

#### The MNS cell lines show distinct expression profiles of region-specific genes

Using these cell lines, we performed intensive analyses of the expression of a series of genes that are restricted to specific domains of the developing forebrain and midbrain, from which all the MNS cell lines originated. First, monolayer culture of each cell line was subjected to quantitative RT-PCR analysis. As shown in Fig. 5A, a high level of *Pax-3* expression was detected only in MNS-71 cells, which was about four times higher than that in the mixture of forebrain and midbrain neuroepithelium and comparable to that in the caudal midbrain-enriched preparations (see Table 2). In contrast, the levels of

the *Pax-3* transcript in the other four cell lines were less than 3% of the control. Likewise, a significant level of *Pax-5* expression was detected only in MNS-57 cells. Although *Pax-6* expression was observed in all the cell lines, the levels were highly variable among them. For example, a 60 times higher level of *Pax-6* transcript was detected in MNS-70 than in MNS-92 cells (see Table 2 for results of quantification).

We also examined the expression of four families of the homeobox genes (Fig. 5B). With respect to the *Dlx* family, all the cell lines expressed detectable levels of *Dlx-1* and *Dlx-2* transcripts. However, more than 100-fold differences were observed among different cell lines; MNS-71 cells expressed the highest level (nine times higher than the E11.5 control tissue for *Dlx-1*, and four times for *Dlx-2*), whereas MNS-8



**Fig. 4.** Induction of neuron- and glia-specific marker genes upon induction of differentiation of MNS cell lines. RT-PCR analysis of the induction of neuronal (MAP2), astroglial (GFAP), and oligodendroglial (CNPII) markers in MNS-8 cells. A shows photographs of PCR products stained with 0.01% SYBR Green I. The positions of molecular size markers (MW) are shown on the left, where arrowheads indicate the ladder of  $\lambda$ -X174 DNA digested with *Hae*III (from the top, 1078 bp, 872 bp, 603 bp, and triplet of 310/281/271 bp). The sizes of specific PCR products are: 405 bp for MAP2; 508 bp for GFAP; and 450 bp for CNPII (see Table 1). Broad bands at the bottom of each figure represent the unincorporated PCR primers. Values for fold-induction of these genes were measured as described in Fig. 2 and are shown in B, where the level in monolayer cells was designated as 1.0. Open bars, monolayer culture (M); hatched bars, aggregation culture (A); and closed bars, differentiation culture (D). The cycle numbers of PCR reactions used for visualization (A) and quantification (B) were: 32 for MAP2; 30 for GFAP; and 32 for CNPII.

cells expressed less than one fifth of the control (Table 2). As shown in Fig. 2, *Dlx-1* and *Dlx-2* transcripts were most abundant in the ventral forebrain, and their levels were 8 to 17 times higher than those in our mixed preparation of E11.5 forebrain and midbrain cells (Table 2). Therefore, levels of *Dlx-1* and *Dlx-2* in MNS-57 and MNS-71 cells are comparable to those in 'Dlx-high cells' present in the ventral forebrain in vivo. Moreover, it is notable that *Dlx-1* and *Dlx-2* showed parallel expression patterns among different cell lines, which coincides well with their in vivo expression patterns (Bulfone et al., 1993).

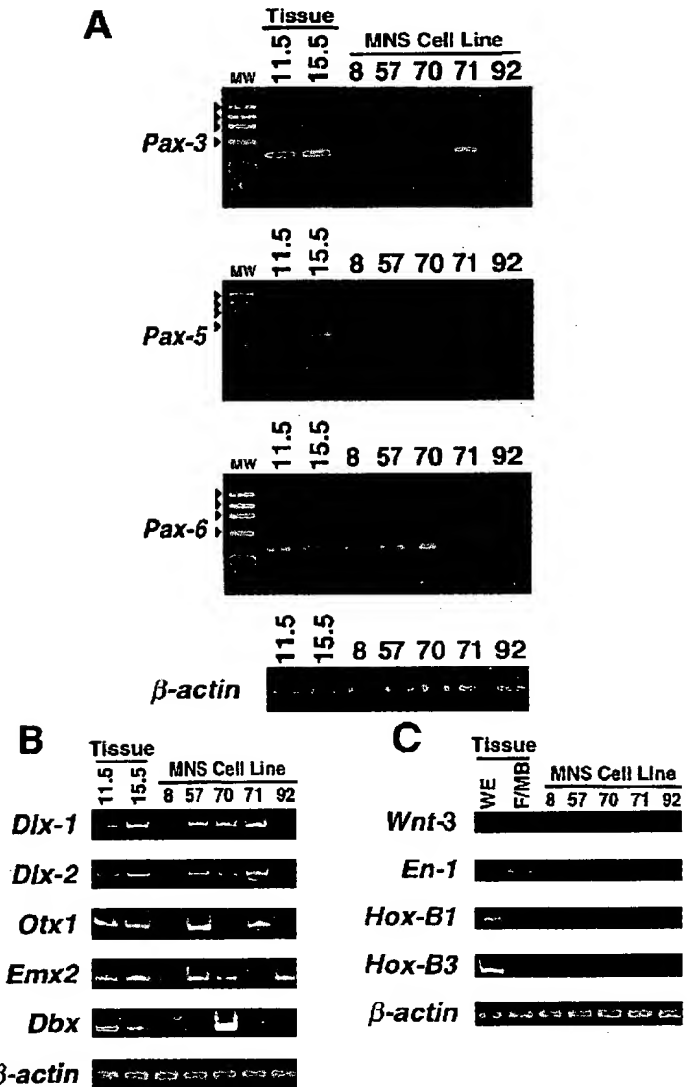
Members of the *Otx*, *Emx* and *Dbx* families also showed differential expression patterns among the five cell lines. RT-PCR and sequencing analyses revealed that among the members of these families, expression of only *Otx1*, *Emx2* and *Dbx* could be detected in the MNS cell lines. As shown in Fig. 5B and Table 2, *Otx1* and *Emx2* transcripts were detectable in all the cell lines, but there were marked differences in expression levels. The *Dbx* gene was expressed only in MNS-57, -70 and -71 cells. Again, quantitative comparison with primary tissue samples established that the expression levels of these genes in MNS cell lines were within the range of physiological levels.

Although all the genes described above have restricted expression domains in the developing forebrain and midbrain, some are also expressed in more caudal regions of the neural tube. Thus, we also examined the expression of four other genes that are expressed only in the caudal regions of the brain; *Wnt-3* (Bulfone et al., 1993; Salinas and Nusse, 1992), *En-1* (Davis et al., 1991), *Hox-B1* and *Hox-B3* (Wilkinson et al., 1989a; Lumsden, 1990). Transcripts of these genes were all undetectable in the MNS cell lines (Fig. 5C).

In summary, distinct MNS cell lines expressed different repertoires of region-specific genes, but they were restricted to those found in specific domains of the forebrain and/or midbrain in vivo. Thus, the overall expression patterns are consistent with the forebrain/midbrain origins of the cell lines. Table 2 summarizes the expression profiles of region-specific genes in the five MNS cell lines. It should be noted that each cell line was derived from a single neuroepithelial cell and was clonally expanded. We confirmed that the gene expression pattern observed in each cell line was strictly maintained during repeated passages of the cells. Thus, it is concluded that MNS cell lines possess certain cell-autonomous mechanisms that maintain the expression of specific sets of region-specific genes in vitro even in the absence of environmental signals.

#### The expression of region-specific genes is maintained during proliferation and differentiation of MNS cell lines

We expected that if cell-autonomous mechanisms are operating independent of environmental signals, a particular set of genes expressed in undifferentiated MNS cells would be maintained even when the cells undergo proliferation and differentiation. To



**Fig. 5.** Differential expression of various region-specific genes in monolayer cultures of MNS cell lines. The levels of expression of the *Pax* genes (A) and the four families of homeobox genes (B) were examined among different MNS cell lines cultured in monolayer as described in detail in Materials and Methods. Expression levels of each gene in the mixed preparation of the forebrain and midbrain tissues from E11.5 and E15.5 embryos are also shown as controls. In panel A, the positions of molecular size markers (MW) are shown by arrowheads on the left as described in Fig. 4A. The sizes of specific PCR products are: 418 bp for *Pax-3*; 447 bp for *Pax-5*; and 405 bp for *Pax-6*. Broad bands at the bottom of each figure represent the unincorporated primers. In panel B, weakly stained and faster migrating bands in the lanes for *Dlx-2* show minor nonspecific PCR products, which was confirmed by subcloning and sequencing. See Table 2 for the results of the quantification and PCR cycle numbers used for each gene. In the experiments in Fig. 5, for the clearer visualization of the products, we used cycle numbers 2-3 times larger than those used for quantification. Photographs were obtained directly from the image files generated in the fluorescence image analyzer. The data shown are representative of three to five independent experiments. (C) Results of RT-PCR analyses of *Wnt-3*, *En-1*, *Hox-B1* and *Hox-B3* expression are shown. In all experiments, total RNA from monolayer culture of each cell line was used. The control cDNAs were prepared from E11.5 whole embryos deprived of the forebrain and midbrain (WE) and E11.5 forebrain and midbrain (F/M B), respectively. Note that transcripts of these genes were undetectable in MNS cell lines after 40 cycles of PCR amplification, and their levels were estimated to be at least 100 times lower than those in the control samples.

examine this possibility, we carried out RT-PCR analyses using RNA from cells cultured under three different (monolayer, aggregation and differentiation) conditions. As described above, monolayer culture represents conditions under which undifferentiated MNS cells undergo clonal expansion. In aggregation culture, cell growth was stimulated by the growth factor bFGF and --E2, an activator of the c-MycER protein, and we assume that during this period the cells undergo commitment to become neurons and glia. In differentiation culture, differentiated neurons and glia were generated, and there were no overall increases in cell number. Representative growth properties of MNS-71 cells are shown in Fig. 6. Similar growth and differentiation patterns were observed in all the MNS cell lines examined in this study (for more detailed description, see Nakafuku and Nakamura, 1995). Furthermore, kinetics of the expression of lineage-specific marker genes clearly showed that differentiation of the MNS cell lines can be conditionally induced (Fig. 4). Thus, this three-step culture protocol allowed us to examine the regulation of region-specific genes in association with proliferation and differentiation of MNS cell lines.

In MNS-71 cells, a high level expression of *Pax-3* remained almost unchanged when proliferation was stimulated in aggregation culture (Fig. 7C). However, its expression level was about three times higher in differentiation culture, where the cells differentiated and ceased proliferation (Figs 6C, 7C). With respect to the *Pax-6* gene, strong (more than 20-fold) induction of expression was observed upon differentiation of MNS-8 cells (Fig. 7A). Weaker but significant upregulation of *Pax-6* was also evident in MNS-70 and -71 cells. Previous studies in mice (Walther and Gruss, 1991) and zebrafish (Macdonald et al., 1994) have revealed that *Pax-6* is expressed

in a subset of differentiated neurons. Thus, we asked whether neurons generated from MNS cells express *Pax-6*. As shown in Fig. 8, specific antibodies identified Pax-6 proteins in MAP2-positive neurons in differentiation culture of MNS-70 cells. The expression of two homeobox genes, *Dlx-1* and *Otx1*, was also examined in MNS cell lines. In the three cell lines which we examined, both *Dlx-1* and *Otx1* expression was maintained and their levels increased upon proliferation and differentiation of the cells (Fig. 7). Robel et al. (1995) recently reported that bFGF upregulates the expression of *Otx2* in primary culture of neuroepithelial cells. In our study, proliferation of MNS cell lines were also stimulated by bFGF in aggregation culture, which in many cases, led to the upregulation of the mRNA levels of most of the above genes. Thus, these results may suggest some common regulatory mechanisms for various region-specific genes related to the growth of neuroepithelial cells.

In contrast to the above results, genes that were not expressed in monolayer cultures did not show detectable induction during aggregation or differentiation culture of MNS cell lines. For example, *Pax-3* expression, which was undetectable in monolayer cultures of MNS-8 or -70, was not induced at detectable levels even after aggregation or differentiation (Fig. 7A,B). Similar results were obtained for all the other genes examined in this study (data not shown). Furthermore, it is notable that although the expression of various region-specific genes examined were all upregulated upon induction of differentiation, the overall expression profile in monolayer culture of each cell line was conserved in this process. For example, in MNS-71 cells, high levels of expression of *Pax-3* and *Dlx-1* were maintained among three culture conditions, while the levels of *Pax-6* and *Otx1*, which were relatively lower than

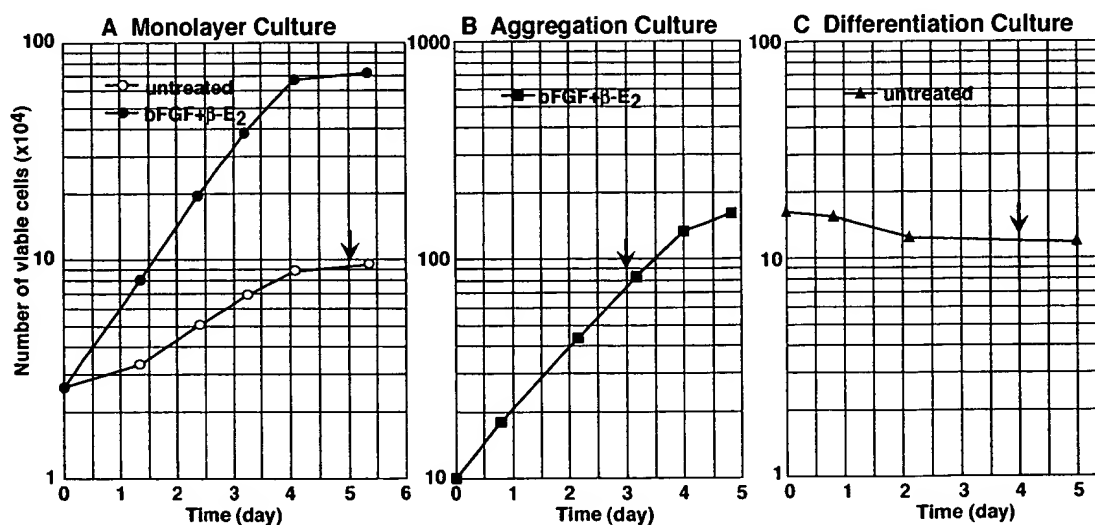
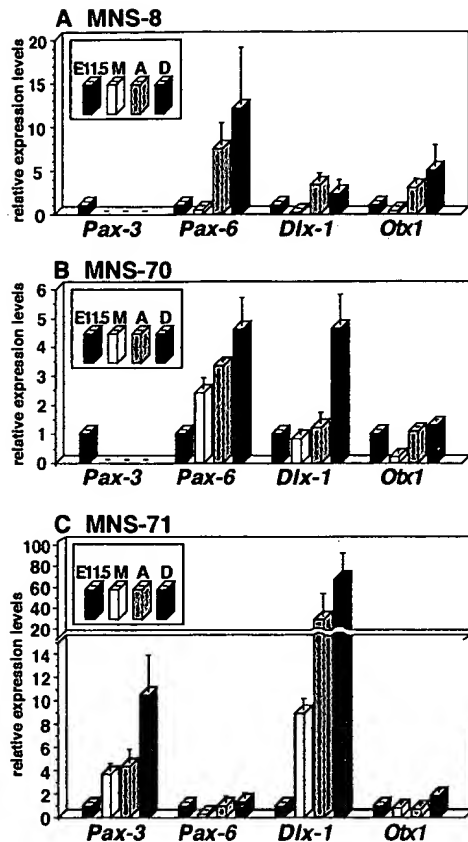


Fig. 6. Growth properties of MNS-71 cells under different culture conditions. MNS-71 cells were cultured under the three different conditions (monolayer, aggregation, and differentiation culture, for details, see Materials and Methods), and the growth of the cells was examined. For the monolayer culture (panel A), the cells were seeded at a density of  $1 \times 10^4$  cells per ml in DF medium containing 10% FBS and 5% HS. Twenty-four hours later, the medium was replaced by the above medium supplemented with (closed circles) or without (open circles) 20 ng/ml bFGF and 1  $\mu$ M --E2, and the subsequent increase in cell number was monitored daily. For the aggregation culture (B), cell suspensions at a density of  $1 \times 10^5$  cells per ml were seeded onto poly HEMA-coated dishes in the above medium plus bFGF and --E2. For the differentiation culture (C), the cells aggregated in the presence of bFGF and --E2 for 3 days were re-seeded onto poly-D-lysine-coated dishes, and incubation was continued in DF medium plus 10% FBS without bFGF or --E2. The arrow in each panel indicates the time when the cells were harvested and subjected to RNA preparation.



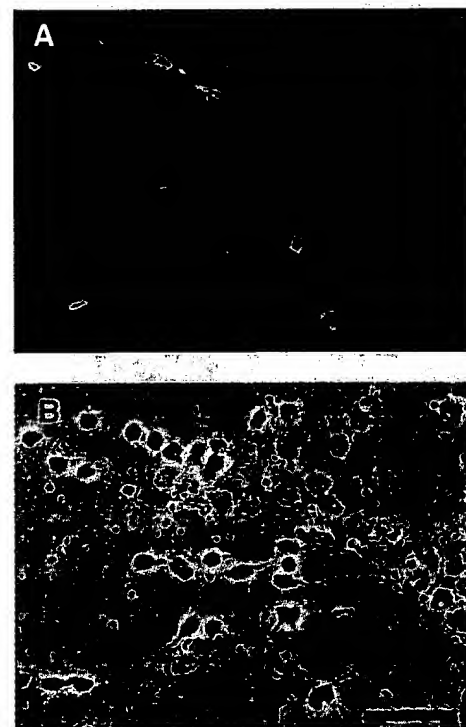
**Fig. 7.** Regulation of *Pax-3*, *Pax-6*, *Dlx-1* and *Otx1* expression upon differentiation of MNS cell lines. MNS cell lines were cultured under three different conditions as described in Fig. 3. Total RNAs were isolated, and relative expression levels of region-specific genes in MNS-8 (A), MNS-70 (B) and MNS-71 (C) were quantified as described in Fig. 2. In each set of genes and cell lines, the expression level in the forebrain and midbrain neuroepithelia from E11.5 embryos (E11.5; solid bar) was designated as 1.0, and those in monolayer (M, open bars), aggregation (A, light shaded bars) and differentiation (D, dark shaded bars) cultures were shown as mean ( $\pm$ s.d.,  $n=3-5$ ) values. The cycle numbers used for quantification were the same as those described in Table 2.

those in other cells, remained low under different culture conditions. Likewise, MNS-70 maintained the pattern of high expression of *Pax-6* and *Dlx-1* and low expression of *Pax-3* and *Otx1* under all culture conditions. This general feature is consistent with the data obtained in primary culture of neuroepithelial cells shown in Fig. 2, in which undifferentiated neuroepithelial cells at DIV 0 underwent significant proliferation and differentiation during 5 days in culture. Thus, the most important conclusion from these results is that the differential expression patterns of region-specific genes in undifferentiated MNS cell lines are maintained even after induction of their proliferation and differentiation.

#### SHH induces differential expression of ventral marker genes in MNS cell lines

We next asked whether distinct cell-autonomous properties among different neuroepithelial cells influence the responsive-

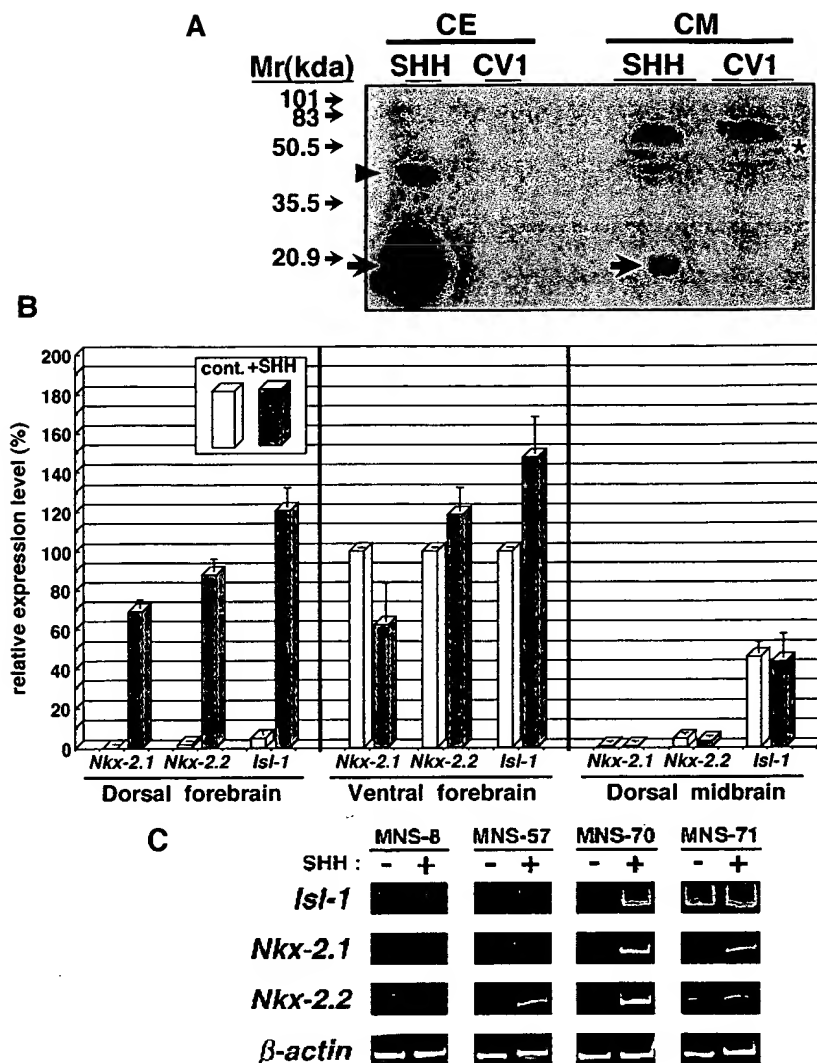
ness to environmental signals. For this purpose, we examined their responses to SHH, which is one of the best characterized signaling molecules involved in regional specification. It has been shown that SHH induces the expression of a series of ventral cell-specific transcription factors including *HNF-3*-, *Isl-1*, *Nkx-2.1*, *Nkx-2.2* and *Lim-1*, thereby playing a crucial role in specification of the ventral phenotype of neuroepithelial cells (Echelard et al., 1993; Roelink et al., 1994; Martí et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995). In this study, we utilized CV1 cells which express chicken SHH at high levels and secrete its N-terminal cleavage product in the conditioned medium (Ogura et al., 1996; Fig. 9A). We first isolated primary cultures of neuroepithelial cells from three distinct regions, and compared their expression levels of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes (Fig. 9B). Consistent with previous in situ studies in chick and mouse embryos (Ericson et al., 1995; Shimamura et al., 1995), *Nkx-2.1* expression was restricted in the ventral forebrain, and its levels in the dorsal forebrain and midbrain were less than 0.3% and 0.1% of that in the ventral forebrain, respectively. The expression of *Nkx-2.2* and *Isl-1* was detected both in ventral forebrain and midbrain preparations, but they were much weaker in the dorsal forebrain (1.3% and 4.5 % of that in the ventral forebrain for *Nkx-2.2* and *Isl-1*, respectively). Upon incubation with the medium conditioned by CV1SHH cells, the levels of the all



**Fig. 8.** The *Pax-6* protein was expressed in MAP2-positive neurons generated from MNS-70. MNS-70 cells grown under differentiation culture conditions were analyzed by double immunostaining with anti-*Pax-6* and anti-MAP2 antibodies. Secondary antibodies used were FITC-conjugated anti-mouse IgG for *Pax-6* and TR-conjugated anti-rabbit IgG for MAP2. (A) Fluorescence micrograph showing the coexpression of *Pax-6* (green) and MAP2 (red). Cells that expressed both proteins are shown in yellow. (B) Phase contrast micrograph showing the same field as A. Scale bar, 100  $\mu$ m.

three genes in the dorsal forebrain was dramatically elevated and were close to those of the ventral forebrain (Fig. 9B). These results are consistent with the idea that SHH has the ability to ventralize the dorsal neuroepithelium (Ericson et al., 1995; Lumsden and Graham, 1995). In the ventral forebrain, which endogenously expressed much higher levels of *Nkx-2.2* and *Isl-1*, they remained almost unchanged after the treatment with exogenous SHH. This is probably due to the presence of large numbers of SHH-expressing cells in this preparation. Dorsal midbrain cells expressed *Nkx-2.1* and *Nkx-2.2* at very low levels, and exogenous SHH did not increase the levels of either genes significantly. In particular, the absence of *Nkx-2.1* induction in the midbrain and its remarkable induction in the dorsal forebrain coincided with its forebrain-specific expression in vivo (Shimamura et al., 1995). It is also consistent with a previous report describing that SHH could induce *Nkx-2.1*-positive cells in explant culture of the forebrain neuroepithelium but not of the hindbrain (Ericson et al., 1995). These results indicate that neuroepithelial cells in distinct regions possess distinct properties in terms of the expression of ventral-specific genes and their responsiveness to SHH.

Next we asked how clonal MNS cell lines respond to SHH. Among the monolayer cells of MNS-8, -57, -70, -71 and -92, *Isl-1* was expressed only in the MNS-71 cells (the relative level of expression was  $0.77 \pm 0.39$ , where that of the control E11.5 forebrain/midbrain tissue was designated as 1.00,  $n=3$ ), and *Nkx-2.2* was expressed only in MNS-71 cells ( $0.33 \pm 0.08$ ,  $n=3$ ; also see Fig. 9C). In all the other cases, expression levels of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* were less than 5% of the control tissue. Furthermore, none of the cell lines expressed *HNF-3*, *Lim-1*, or *SHH* at a detectable level (data not shown). When MNS cell lines were grown in contact with CV1SHH cells, they showed differential induction profiles of the above genes (Fig. 9C). Upon contact with the CV1SHH cells, MNS-70 cells exhibited clearly elevated (more than 50- to 100-fold) levels of expression of *Isl-1*, *Nkx-2.1* and *Nkx-2.2*, which were comparable to those detected in the E11.5 forebrain/midbrain neuroepithelium ( $0.39 \pm 0.06$  for *Isl-1*,  $0.58 \pm 0.14$  for *Nkx-2.1* and  $2.03 \pm 0.45$  for *Nkx-2.2*,  $n=3$ ), although the accurate values for fold-induction were uncertain because of the very low levels of their



**Fig. 9.** SHH induced expression of ventral neuroepithelium-specific genes in MNS cell lines. (A) Western blot analysis of SHH expressed by CV1SHH cells. Whole cell extracts (CE) and conditioned media (CM) were prepared from  $5 \times 10^3$  cells of the control and CV1SHH. These samples were subjected to 15% polyacrylamide gel electrophoresis and blotted with the anti-SHH antibody. The arrowhead and arrows with apparent relative molecular masses of 46–103 and 20–103 correspond to the full-length and its amino-terminal cleavage products of chicken SHH, respectively. The nonspecific bands in lanes of CM (marked by an asterisk) were due to the presence of large amounts of bovine serum albumin in the culture medium. (B) Primary culture of neuroepithelial cells was established as described in Materials and Methods, in which cells of three distinct regions, the dorsal forebrain, ventral forebrain, and dorsal midbrain were isolated separately. Note that in these experiments, the ventral two-thirds was removed from the caudal midbrain preparation used in Fig. 2. Subsequently, the cells were treated with the medium conditioned by the control CV1 (cont., shown by open bars) or CV1SHH (+SHH, shaded bars) cells for 3 days, and RNA was prepared for quantitative RT-PCR analysis of the *Isl-1*, *Nkx-2.1* and *Nkx-2.2* genes. For each gene, relative expression levels are shown as mean  $\pm$  s.d. of three independent experiments, where the expression level in the ventral forebrain was designated as 100. (C) RNAs were prepared from the MNS cell lines cultured for 3 days in contact with control (–) or CV1SHH (+) cells. The induction of *Isl-1*, *Nkx-2.1* and *Nkx-2.2* was analyzed by quantitative RT-PCR. The cycle numbers of PCR reactions for Panels B and C were: 29 for *Isl-1*; 35 for *Nkx-2.1*; 32 for *Nkx-2.2*; and 20 for  $\beta$ -actin.

expression in the control (SHH-untreated) cells. MNS-71 cells showed a very weak but detectable induction of *Nkx-2.1* in response to SHH, but the expression of *Isl-1* and *Nkx-2.2*

remained almost unchanged ( $0.69 \pm 0.15$  for *Isl-1*, and  $0.57 \pm 0.26$  for *Nkx-2.2*,  $n=3$ , where the levels in the control cells were designated as 1.00). MNS-57 cells underwent significant induction of the *Nkx-2.2* expression (the level in the SHH-treated cells was  $0.26 \pm 0.12$  compared with 1.00 of the E11.5 sample,  $n=3$ ) but not of *Isl-1* and *Nkx-2.1*, whereas none of these genes were induced in MNS-8 cells. In contrast, we did not observe detectable induction of *HNF-3* or *Lim-1* in any of these cell lines (data not shown). Similar differential induction patterns were obtained by using the conditioned medium from CV1SHH cells but not with that from the parental CV1 cells (data not shown), indicating that the induction of the ventral genes directly resulted from exposure to SHH. These results demonstrate that distinct MNS cell lines, which maintain the expression of different sets of region-specific genes in vitro, show specific and differential responsiveness to the same inductive signal from the environment, i.e. SHH.

## DISCUSSION

The generation of neural cell diversity in the developing central nervous system is thought to be regulated by both cell-intrinsic and -extrinsic mechanisms (see Jessell and Dodd, 1992; Ruiz i Altaba, 1994; Simon et al., 1995; Lumsden and Graham, 1995 for discussion). However, it is not fully understood how these two mechanisms contribute to the determination of a particular fate of each progenitor cell. Recently, a number of molecules have been identified that potentially regulate this complex process. These genes, collectively called region-specific genes, are expressed in the developing neuroepithelium in a spatially and temporally restricted manner, and have been implicated in the specification of particular domains or layers of the brain. Thus, studies on the regulation of these genes would provide crucial information to facilitate understanding of the molecular nature of the above two mechanisms. Recent studies have uncovered the important roles of inductive signals from the environment for the regulated expression of region-specific genes (Jessell and Dodd, 1992; Johnson and Tabin, 1995), but little is known about the involvement of the cell-autonomous mechanisms. In this study, we have established in vitro culture systems which enable us to study how cell-autonomous mechanisms and environmental signals contribute to the regulation of region-specific genes in neuroepithelial cells.

First, we showed that neuroepithelial cells from distinct regions of the brain express region-specific genes, including *Pax-3*, *Pax-5*, *Pax-6*, *Dlx-1*, *Dlx-2*, *Emx2* and *Dbx* at different levels. The overall pattern in each preparation was generally consistent with the results of previous in situ hybridization studies (Fig. 2). In addition, when cultured for 5 days free from the influences of cells in other regions, cell populations did not undergo significant changes in the overall gene expression pattern. These results support the notion that certain cell-autonomous mechanisms play an important role in maintaining the expression of particular sets of genes in neuroepithelial cells.

Next, we demonstrated that differential expression of region-specific transcription factors among different neuroepithelial cells can be reproduced in immortalized cell lines.

MNS cell lines used in this study, which were established from E11.5 rat forebrain and midbrain (Nakafuku and Nakamura, 1995), shared properties of neural stem cells in that they expressed neuroepithelium-specific antigens and could generate neurons and glia under particular culture conditions (Figs 3, 4). Since MNS cell lines are derived from clonally distinct cells, they provide a useful model system in which to examine what types of region-specific genes are expressed in single neuroepithelial cells, and to study how they are regulated under conditions in which the influence of environmental signals and cell-cell interactions among heterogeneous cell populations can be eliminated. We found that five distinct MNS cell lines expressed different combinations of transcription factors expressed in restricted regions of forebrain and midbrain. In contrast, region-specific genes that are expressed only in the caudal brain, including *Wnt-3*, *En-1*, *Hox-B1* and *Hox-B3*, were all below detectable levels in these cell lines. These results are consistent with the forebrain/midbrain origin of the MNS cell lines, and it is unlikely that the observed gene expression profiles of the MNS cell lines have resulted from some random events related to immortalization or in vitro culture. Furthermore, we demonstrated that these expression profiles remained essentially unchanged upon proliferation and differentiation (see Fig. 7). Immunocytochemical analysis demonstrated that when MNS-70 cells, which expressed the highest level of *Pax-6* among the cell lines examined, were induced to differentiate, *Pax-6*-positive neurons were indeed generated (Fig. 8). These results strongly suggest that at least in some cases, expression of region-specific genes in undifferentiated neural stem cells is directly inherited to their neuronal (and possibly also glial) progeny.

In relation to the above results, comparison of our data shown in Fig. 2 and Table 2 with the available information from a number of previous in situ hybridization studies (Bulfone et al., 1992; Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Rubenstein et al., 1994 for details) demonstrated that the overall gene expression patterns found in some of the MNS cell lines closely matched those in particular regions of the developing brain. For example, MNS-70 cells expressed significant levels of *Pax-6*, *Dlx-1*, *Dlx-2* and *Dbx*, but not *Pax-3* or *Pax-5*. This profile is reminiscent of that in the septal region of the forebrain. However, MNS-8 cells shared similar expression patterns with the dorsal telencephalic region shown in Fig. 2 in that they expressed *Pax-6*, *Otx1* and *Emx2* at significant levels but not *Dlx-1*, *Dbx*, *Pax-3*, or *Pax-5*. MNS-57 cells expressed all the genes examined except *Pax-3*, but the relatively high levels of *Dlx-1*, *Dlx-2* and *Emx2* suggest its ventral forebrain origin, although our data shown in Fig. 2 did not clearly distinguish between the ventral and dorsal forebrain. We consider that these results may suggest the intriguing possibility that each MNS cell line inherited a particular regional identity from the neuroepithelial cell from which it originated. It should be noted, however, that although our data in Fig. 2 clearly showed differential expression profiles of various region-specific genes among distinct brain regions in vivo, we still do not know their exact expression patterns in particular single cells in a given region. For example, it is possible that even in the ventral forebrain where *Dlx-1* is strongly expressed, some cells express it at only very low levels. Thus, the comparison of the combinations of expressed genes alone is not enough at present to definitively

assign the position where each cell line was derived. Nevertheless, strict maintenance of gene expression profiles in MNS cell lines upon continuous cell growth and differentiation makes it likely that a particular set of genes expressed in individual cell lines reflect their distinct origins.

Based on the above results, we can conclude that certain cell-autonomous mechanisms play important roles in maintaining the expression of a specific set of region-specific genes. Several previous studies have also implicated similar mechanisms for the establishment of regional identity in the developing brain. For example, the limbic system-associated membrane protein (LAMP) is specifically expressed in the limbic cortex but not in other cortical areas (Horton and Levitt, 1988). Transplantation and in vitro culture experiments have shown that its specific expression in the limbic cortex is maintained after isolation from the original environment (Barbe and Levitt, 1991; Ferri and Levitt, 1993). Differential expression of the PC3.1 antigen (latexin), which also revealed regional heterogeneity of the developing cerebral cortex in vivo, was conserved in cultured neuroepithelial cells (Arimatsu et al., 1992). Likewise, chick/quail heterotopic transplantation demonstrated that midbrain and hindbrain neuroepithelia maintained their predetermined fates even after being placed into other brain regions (Nakamura, 1990). These studies collectively support our conclusion that cell-intrinsic mechanisms indeed play important roles in determination of the regional fate of neuroepithelial cells.

Finally, we examined the possible cooperative actions of the cell-autonomous and non-cell autonomous mechanisms in the regulation of region-specific gene expression. We first showed that SHH, which is one of the best characterized inductive signals from the environment (Martí et al., 1995; Roelink et al., 1995; Hynes et al., 1995; Ericson et al., 1995; Barth and Wilson, 1995), induced the expression of various ventral-specific genes, including *Nkx-2.1*, *Nkx-2.2* and *Isl-1* in primary cultures of distinct regions of the embryonic neuroepithelium (Fig. 9B). The dorsal forebrain expressed high levels of all these genes in response to SHH, and its pattern resembled that in the ventral forebrain. This result supports the notion that SHH acts as a major ventralizing signal in the forebrain region. A clear difference between the forebrain and midbrain was evident in that the dorsal midbrain did not express *Nkx-2.1* with or without exogenous SHH. These results were consistent with previous studies showing that neuroepithelial explants, derived from distinct regions, generated different cell types in response to SHH. In spinal cord explants SHH induced *Isl-1*-positive cells with the identity of motoneurons (Martí et al., 1995; Roelink et al., 1995), whereas it generated dopaminergic neurons in midbrain-derived explants (Hynes et al., 1995). On the other hand, SHH specifically induced *Nkx-2.1* and *Lim-1* in a population of forebrain-derived neuroepithelial cells in chick embryos (Ericson et al., 1995). However, as in the case of our study using primary culture of neuroepithelial cells, these studies utilized explant cultures which contained heterogeneous cell populations, and hence the contribution of cell-cell interactions among these populations in each explant has remained unclarified. Furthermore, distinct responsiveness among neuroepithelial cells has not yet been fully characterized at the single-cell level. To address this point, we extended the above observations by examining the properties of MNS cell lines. We demonstrated that the cell lines had the ability

to respond to SHH and expressed these ventral cell-specific genes (Fig. 9C). Furthermore, we found that the combinations of the induced genes differed among different cell lines. These results strongly suggest that cell-intrinsic properties of neuroepithelial cells indeed define the responsiveness to environmental signals, as well as the repertoires of genes expressed in their absence. It is notable that SHH induced *Nkx-2.1* expression in MNS-70 and MNS-71 cells, which is consistent with their forebrain origin as suggested by the expression patterns of other region-specific genes (discussed above), since *Nkx-2.1* was specifically induced in the forebrain as shown in Fig. 9B. In addition, the use of homogeneous cell populations enabled us to clearly conclude that intrinsic properties of the cells themselves but not cell-cell interactions among heterogeneous populations are responsible for specifying how neuroepithelial cells respond to SHH.

In summary, it is highly likely that both cell-autonomous mechanisms and environmental factors contribute cooperatively to the differential and regulated expression of the genes specifying the identities of neuroepithelial cells. Many questions, however, still remain to be answered. At present, we do not know exactly how particular region-specific genes are activated in each cell line, how they are maintained, or how various environmental signals contribute to the initial activation and subsequent modulation of region-specific genes. We propose that the MNS cell lines described here will serve as a useful in vitro model to clarify the above questions at the molecular level. Such studies would provide further insight into the molecular basis of regional specification in the developing brain.

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## Interactive report

## Regional specification of rodent and human neurospheres

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**Abstract**

Neural precursor cells were isolated from various regions of the developing rat and human brain and grown in culture as aggregates termed neurospheres. We asked whether cells within human and rodent neurospheres are identical, or whether they have species specific characteristics or differences based on their region of origin. Under our culture conditions, rodent neurospheres isolated from the cortex (<sup>ctx</sup>NS) and striatum (<sup>str</sup>NS) grew faster than those from the mesencephalon (<sup>mes</sup>NS), but stopped growing after only eight to ten population doublings. In contrast, human neurospheres under identical culture conditions, continued to grow for over 40 population doublings. Following migration and differentiation of both rodent and human cultures, <sup>ctx</sup>NS and <sup>str</sup>NS generated high numbers of small neurons whereas <sup>mes</sup>NS generated small numbers of large neurons with many long fibres. Only very rare neurons from <sup>mes</sup>NS expressed dopaminergic markers, and thus may require further signals to fully mature. While the rat neurospheres generated high numbers of oligodendrocytes, very few were found to develop from human neurospheres from any region after a few weeks of passaging. FACS analysis revealed a unique population of smaller cells within human <sup>str</sup>NS and <sup>ctx</sup>NS, which appeared to be neuronal progenitors. However, large cells within neurospheres were capable of generating these small neuronal progenitors following further proliferation. Together, our data show that rat and human neurospheres have unique characteristics with regard to growth and differentiation, and that the majority of precursor cells within neurospheres are regionally specified to generate set numbers of neurons. These findings have important implications for understanding the nature of proliferating neural precursors isolated from the developing CNS, and their potential for brain repair. © 2002 Elsevier Science B.V. All rights reserved.

**1. Introduction**

Small populations of stem cells exist in the developing and adult rodent brain, which can generate progenitor cells capable of differentiating into neurons, astrocytes or oligodendrocytes [17]. However, fundamental challenges within this field of biology are (i) to establish how cell-autonomous programs interact with environmental signals to direct the phenotypic fate of these cells and (ii) to understand the mechanisms underlying their self-renewal capacity [2]. One technique for growing cells derived from the germinal zones of either the developing or adult CNS involves the generation of free floating spherical aggre-

gates termed 'neurospheres'. This method was developed for rodent tissues a number of years ago [32] and has recently been adapted for the long-term growth of human neurospheres by ourselves and others [10,45,49]. Neurospheres from rodents consist of both multipotent stem cells and more restricted progenitors [33] and, as such, are considered to comprise a heterogeneous population of neural precursor cells (NPCs) [41]. Although the selection of sphere forming cells from primary neurogenic zones within differentiating CNS regions of the animal is possible [34,47], the reliable distinction between true stem cells and more restricted progenitors within expanded populations of neurospheres has been limited by the lack of available cell-type specific markers. It is possible that such cells may be regionally specified. If this were true, neurospheres generated from different brain regions would retain some features of this region, even following expansion in culture. Alternatively, is it possible that a common

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stem cell exists along the entire extent of the neuroaxis which, following isolation, would behave in a similar fashion irrespective of its origin. In addition to these regional complexities, there are likely to be differences between species with regard to general stem cell biology that need to be addressed, particularly in the context of potential clinical applications.

Clearly, tissues derived from one brain region and expanded in culture can take on the phenotype of another following transplantation [16,36,40] or, in more extreme cases, can trans-differentiate into cells of different dermal origin when injected into irradiated mice or blastocysts [5,12]. These results suggest that at least some neuro-epithelial cells are extremely plastic and environmentally specified, with very little evidence of genetic determination. However, these types of cells may be only a very small fraction of cells within such cultures. Although cells derived from different embryonic brain regions and expanded in culture adopt host region phenotypes when transplanted, there is evidence that some of these cells retain a molecular memory of where they came from, based on the expression of regionally expressed genes [28,35,53] or proteins [14]. In addition, although cells derived from the embryonic forebrain migrate and differentiate within hind-brain regions when transplanted into neonates [9], these cells continue to express markers associated with their region of origin [27]. Very recently, the region specific differentiation of spinal cord progenitor cells [52] and mammalian neural crest cells [51] has also been reported following transplantation. Thus, cell autonomous mechanisms may exist to control the fate of these cells. Neurospheres generated from the human fetal brain produce large number of neurons [42], but those from the spinal cord exclusively produce astrocytes using slightly different growth conditions [3,31], suggesting that some regional specification also exists along the human neuroaxis. However, a direct comparison of proliferative and phenotypic potential of neurospheres generated from different brain regions and grown under identical culture conditions, has not previously been undertaken.

To address these issues, we compared the growth and differentiation of non-genetically modified epidermal growth factor (EGF)- and fibroblast growth factor-2 (FGF-2)-responsive neural precursors isolated from various regions of the embryonic rat and human brain.

## 2. Materials and methods

### 2.1. Rodent neural precursor cell cultures and proliferation studies

The cortex, striatum (comprising both medial and lateral ganglionic eminences) and ventral mesencephalon (VM) were dissected from embryonic day 14 (E14) rat brain. Human embryonic tissue (between 6 and 21 weeks post

conception) was collected following routine terminations of pregnancy. The methods of human tissue collection conformed with the arrangements recommended by the Polkinghorne Committee for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health. The same regions plus the cerebellum and thalamus were isolated from human fetal samples. Tissue was treated with trypsin (0.1% for 20 min), washed in DMEM and then dissociated into a single cell suspension. Cells were initially seeded at a density of 400,000 per ml into T75 flasks containing 20 ml of defined serum-free medium (DMEM:HAMS-F12 at 3:1) supplemented with B27 (2% v/v), epidermal growth factor (EGF, 20 ng per ml) and fibroblast growth factor (FGF-2, 20 ng per ml) with heparin (5 µg per ml).

Cells from all regions of both the rat and human tissue formed neurosphere cultures during the first 2–5 days of growth. Due to differences in expansion rates between the rat and human cells, they were then passaged differently from this point. The rat cultures were passaged at 7 days (P1) and then every 14 days (P2, P3) by chopping spheres into 200-µm sections, which were then re-seeded into fresh growth medium containing both EGF and FGF-2 and B27 at a density equivalent to ~200,000 cells per ml. The sectioning of neurospheres has previously been developed as a method for optimising the *in vitro* expansion of human NPCs [45]. Estimates of rat cell expansion were undertaken every 7 days by the removal of aliquots of cells from the flasks, which were then dissociated and counted using the trypan blue exclusion method. To establish a relationship between cell number and neurosphere volume, sequential measurements of sphere size were undertaken for individual rat neurospheres between 7 and 16 days in culture.

The human neurospheres were passaged every 14 days by sectioning of spheres into 350-µm sections, that were re-seeded into fresh growth medium at a density equivalent to 200,000 cells per ml. Half the growth medium was replenished every 4th day. Passaging of cells was undertaken every 14 days. After the first passage all cells were grown in EGF and FGF-2 supplemented media, but B27 was replaced with the supplement N2 (1% v/v, Gibco). At 4 weeks of growth all cultures were switched to EGF alone and fed every 4 days and passaged every 14 days thereafter. These slight differences to the rodent cultures were introduced because the human cultures could be expanded for long periods of time without the addition of either B27 or FGF-2 and these factors were not required for continual growth of the human cells for up to 250 days [45].

### 2.2. *In vitro* differentiation studies and neuronal quantification

We used a cell migration assay, which has been previously described in detail [7], to assess the differentiation potential of neurospheres grown from either the rat or

human. Whole neurospheres generated from the different CNS regions and at sequential passages were plated directly onto poly-L-lysine/laminin-coated glass coverslips in serum-free medium (DMEM:HAMS F-12) containing B27 supplement (2% v/v) but without mitogens. Over a 7–14-day period following plating, cells migrated away from the sphere and formed a differentiating neuronal and glial monolayer. The cells were fixed on day 7 or 14 in 4% paraformaldehyde and rinsed in PBS. Fixed cultures were blocked in 3% goat serum with 0.3% Triton X-100 and incubated with primary antibodies to  $\beta$ -tubulin III (monoclonal, 1:500, Sigma), glial fibrillary acidic protein (GFAP; polyclonal, 1:1000, DAKO), Gal-C (monoclonal, 1:300, Sigma) or tyrosine hydroxylase (TH; monoclonal; 1:500, Chemicon). Following rinsing in PBS, the cultures were incubated in either biotinylated goat anti-mouse or fluorescein-conjugated goat anti-rabbit antibodies. Biotinylated cultures were visualized using a streptavidin-rhodamine conjugate, and Hoechst 33258 was used as a nuclear stain. In order to demonstrate that differentiated cells had arisen from dividing NPCs, some neurospheres were pulsed with BrdU (0.1  $\mu$ M) for 12 h prior to plating and differentiation. Following fixation, these cells were then co-stained for BrdU following the protocol supplied by a commercially available kit (Boehringer) and either  $\beta$ -tubulin-III, GFAP or Gal-C.

Quantification of cells migrating out from neurospheres was achieved by viewing cells under a fluorescence microscope ( $\times 40$  objective) and counting Hoechst-stained nuclei along with labelled neurons in at least four independent fields (total area  $>0.25$  mm<sup>2</sup>) immediately adjacent to plated spheres using a pre-defined template. Measurements of neuronal cell body areas were undertaken using Openlab 2.1 digital imaging software.

### 2.3. [<sup>3</sup>H]Thymidine incorporation assay

Single spheres were exposed to 0.5  $\mu$ Ci per ml of [<sup>3</sup>H]thymidine for 24 h at 37°C, in the presence of growth factors as appropriate. At the end of the incubation period spheres were washed three times with DMEM and incubated for 30 min at 4°C with 10% trichloroacetic acid (TCA) to remove free [<sup>3</sup>H]thymidine. The spheres were then rinsed three times with 10% TCA and washed once with 95% ethanol. The incorporated [<sup>3</sup>H]thymidine was solubilized with 0.5 M NaOH for 30 min at 37°C, which was then neutralized by addition of 1 M HCl. This solution was added to 4 ml of scintillation cocktail and counted in a scintillation spectrometer.

### 2.4. FACS analysis

Whole neurospheres, or neurospheres plated for 7 days, were incubated in 0.1% trypsin for 20 min, washed in DMEM and then seeded into L15 medium (Gibco) supplemented with B27 (1:50) and kept at 4°C. The cells were

then incubated in propidium iodide (PI) for 10 min to label dead cells and then filtered through a sterile high pass filter into a FACS analysis tube. The analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon laser emission wavelength of 488 nm as described in detail previously [25]. PI and autofluorescence was identified using a 585-nm band pass filter.

### 2.5. Statistical analysis

All data are expressed as means  $\pm$  S.E.M. and were analysed using two-way ANOVA with Bonferroni post hoc comparisons (GraphPad Prism software version 3.00).

## 3. Results

### 3.1. Regional differences in growth rates of rat neurospheres

Cells derived from either the E14 rat cortex (<sup>ctx</sup>NS) or striatum (<sup>str</sup>NS) grew as neurospheres following plating and showed exponential growth over the first 35 days (Fig. 1A). These numbers represent a nearly 170-fold expansion in cell number, theoretically equivalent to approximately eight population doublings. In contrast to the forebrain neurospheres, those derived from the mesencephalon (<sup>mes</sup>NS) underwent an approximate three- to four-fold expansion (from  $4 \times 10^6$  to  $15.2 \pm 3.2 \times 10^6$ ) over the same period, and there was no further increase in the cell number after 21 days in culture (Fig. 1A). Post hoc comparisons between the groups revealed significant differences between effective expansion ratios for neurospheres derived from the forebrain (cortical or striatal) and midbrain ( $P < 0.05$ ) at 28 and 35 days in culture. We have previously shown that in contrast to mouse neurospheres, human and rat neurospheres could not be expanded for more than 5 weeks in culture [43,44]. We were able to overcome this growth limitation in human neurospheres by developing a chopping method of passaging which maintained cell–cell contact and allowed extended growth of human neurospheres for up to 150 days [45]. However, the same chopping method did not extend the growth of rat neurospheres. All of the rat neurospheres, regardless of regional origin, underwent senescence at 5 weeks (Fig. 1A). These results suggest that there are fundamental differences between rat and human neurospheres with regard to their continual propagation in culture using these methods.

The regional differences in rat NPC growth rates were further reflected in the relative changes in sphere size measured between day 7 and day 16 of culture (Fig. 1B). Between these time-points, the mean neurosphere diameter increased from  $0.33 \pm 0.03$  to  $0.71 \pm 0.06$  mm for <sup>ctx</sup>NS,

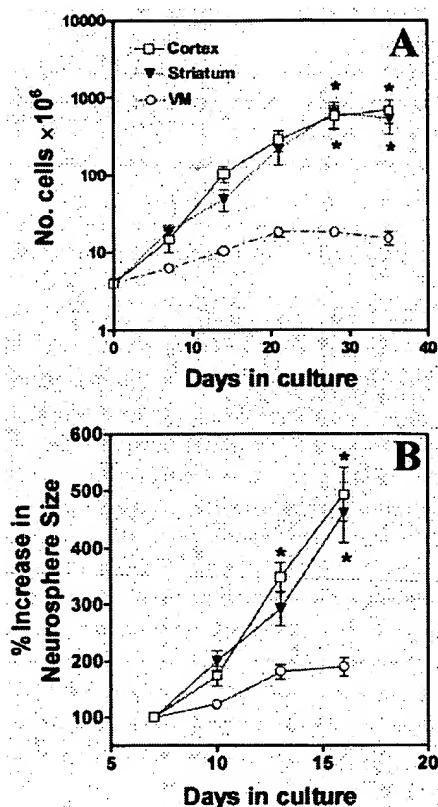


Fig. 1. Cells from the mesencephalon grow more slowly than those from the striatum or cortex. (A) Cell numbers at sequential passages. Data are means  $\pm$  S.E.M. for three independent experiments. Two-way ANOVA revealed a significant overall interaction between the regions across time in culture ( $P < 0.001$ ). Asterisks indicate significant difference in cell numbers between forebrain (cortical or striatal) groups and VM group at 28 and 35 days in vitro ( $P < 0.05$ ). (B) Neurosphere size expressed as percentage increase in sphere area between days 7 and 16 in culture. Data are means  $\pm$  S.E.M. for  $n = 12$  spheres per region. Asterisks indicate significant difference between forebrain (cortex or striatal)-derived neurospheres and VM neurospheres ( $P < 0.05$ ).

from  $0.32 \pm 0.01$  to  $0.65 \pm 0.05$  mm for <sup>str</sup>NS and from  $0.26 \pm 0.01$  to  $0.35 \pm 0.03$  mm for <sup>mes</sup>NS ( $n = 12$  spheres per region). The corresponding percentage increases in sphere

size over a 10-day period also revealed significant differences between <sup>ctx</sup>NS/<sup>str</sup>NS and <sup>mes</sup>NS ( $P < 0.05$ ).

### 3.2. Regional differences in neuronal production for rat neurospheres

Following mitogen withdrawal, whole neurospheres derived from the respective regions of the embryonic brain, and taken at sequential passages, were allowed to differentiate using an established cell migration assay [7]. In the migration assay, individual cells are required to migrate from the plated neurosphere and onto the substrate in order to be analysed. Therefore, the relative proportions of neurons, astrocytes and oligodendrocytes represent those cells that have actively migrated as progenitor cells out onto the substrate and subsequently undergone differentiation, rather than any remaining post migratory cells. After 7 days in vitro, cells that had migrated out from the neurosphere were seen to express  $\beta$ -tubulin-III, GFAP and Gal-C. In BrdU pulse-chase studies, many of the differentiated neural phenotypes could be co-labelled for BrdU suggesting that they had arisen de novo from the proliferating NPCs (Fig. 2). <sup>str</sup>NS gave rise to significantly more neurons than <sup>ctx</sup>NS or <sup>mes</sup>NS at 21 days of expansion ( $P < 0.001$ ) (Fig. 3A,B). Overall, within each region, there was a trend towards a reduction in neuronal emergence with time (passage) in culture, although this result was not statistically significant. At late passages the <sup>mes</sup>NS produced very few neurons. The differences between regions for neuronal differentiation were sustained at sequential time-points in culture (Fig. 3A,B), suggesting that any regionally-defined determinants for neuronal differentiation were conserved despite on-going proliferation ex vivo. All regions produced  $\sim 10$ – $20\%$  oligodendrocytes at each passage, although this was not quantified in detail.

In order to determine whether migrating neurons showed distinct region specific morphologies, we assessed cell body area. Neuronal cell body areas were found to be significantly greater for neurons generated from <sup>mes</sup>NS than for neurons derived from forebrain neurospheres at 7

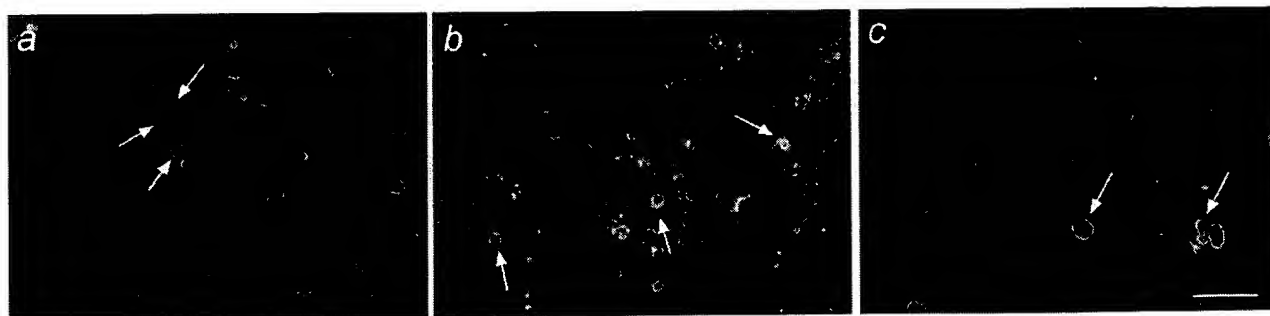


Fig. 2. Fluorescent photomicrographs showing differentiated neural phenotypes (red) emerging from whole neurospheres plated onto PLL/laminin under serum-free conditions:  $\beta$ -tubulin III-positive neurons (a), Gal-C-positive oligodendrocytes (b) and GFAP-positive astrocytes (c). Neurospheres were pre-pulsed with BrdU ( $0.1 \mu$ M for 12 h prior to plating). Cells were fixed at 7 days following plating. Newborn cells arising from proliferating neural precursor cells are double labelled for BrdU (green, arrows). Scale bar represents 50  $\mu$ m.

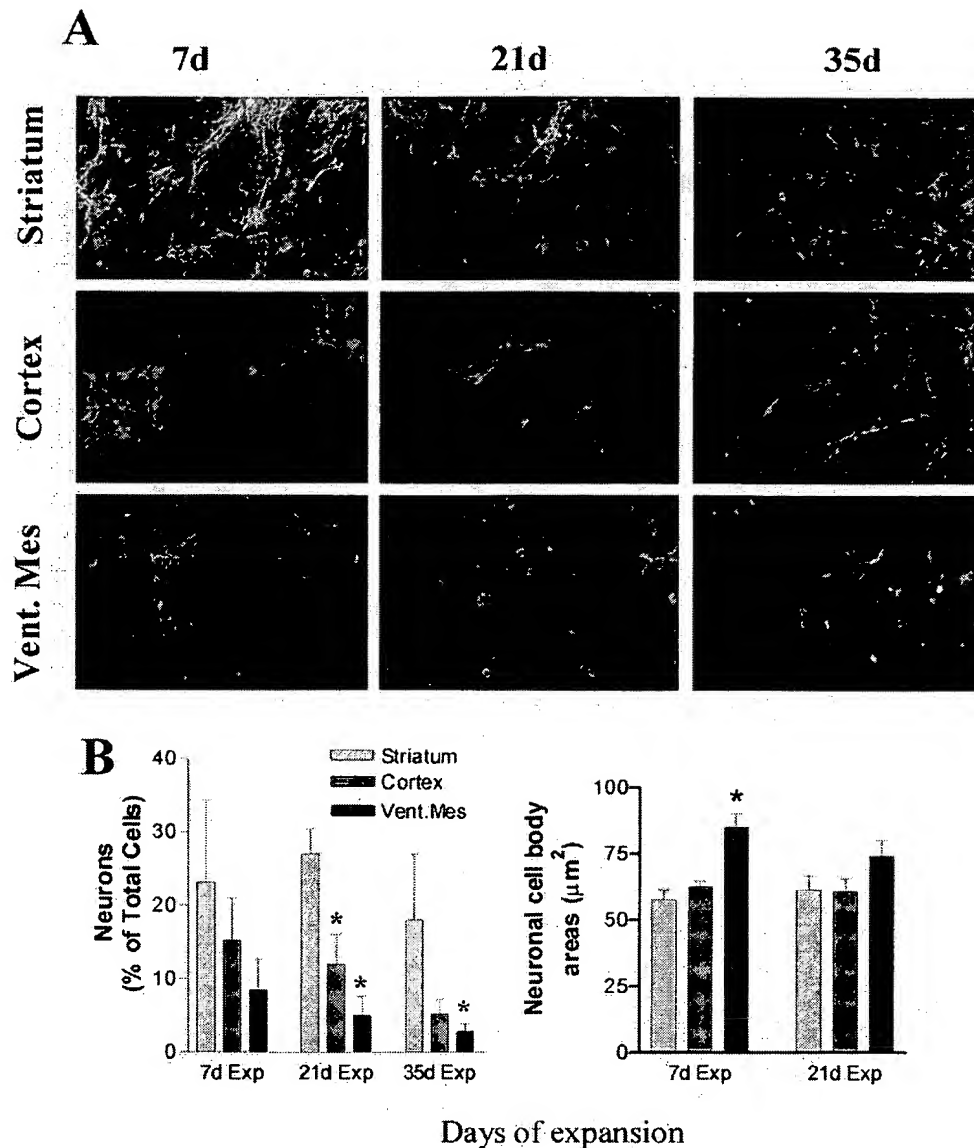


Fig. 3. Rat neurospheres are regionally specified. (A) Following differentiation at each time point, the number and phenotype of cells which had migrated out from spheres were significantly different between the striatum, cortex and mesencephalon. While the <sup>st</sup>NS generated large numbers of neurons, both the <sup>co</sup>NS and <sup>me</sup>NS generated significantly fewer neurons at 21 days, and the <sup>me</sup>NS generated fewer neurons at 35 days (star, significantly different from cortex at  $P < 0.001$ ). (B) There were significantly larger cell bodies for  $\beta$ -tubulin III-positive neurons derived from expanded VM neural precursors when compared to neurons derived from forebrain (either cortical or striatal) precursors (asterisk indicates significance at  $P_0$ ,  $P < 0.05$ ). A trend for this difference was maintained at 35 days ( $P_2$ ). Data represent means  $\pm$  S.E.M. across three separate experiments.

days in vitro ( $P < 0.05$ ), and a trend for this difference was retained even after 21 days in vitro (Fig. 3B). We next assessed whether there were any specific phenotypic markers retained by the VM generated neurospheres. In particular we examined for tyrosine hydroxylase expression, which might indicate dopaminergic differentiation amongst proliferating NPCs. In keeping with our previous report [8] only a small number of well-developed TH-positive neurons were seen when neurospheres were plated following 14 days of growth. Moreover, these cells were found to reside within the limits of the neurosphere and possibly represented primary dopamine neurons which had

not subsequently divided in culture (data not shown). At later passages, it was not possible to identify any TH-immunoreactive neurons in neurospheres generated from any region.

### 3.3. Regional specification of human neurospheres

We have previously shown that neurospheres derived from the human embryonic forebrain can be grown in culture for extended periods of time providing that cell-cell contact is maintained [45]. In the current study, human tissue from all brain regions also produced spheres after

dissociation and seeding into EGF and FGF-2 supplemented culture medium (Fig. 4A). These neurospheres continued to increase in size over time and were passaged using the chopping method. In contrast to the rat neurospheres, human neurospheres derived from all regions showed continual growth over the first 20 weeks of culture, although this was slower than that seen for the rat neurospheres. After 20 weeks of growth there were no significant differences in [ $^3\text{H}$ ]thymidine uptake (a measure of cell proliferation) in neurospheres generated from the different brain regions (Fig. 4B). However, there was a consistent trend for the  $^{\text{mes}}$ NS to grow at a slower rate than those from other regions. Upon mitogen removal and exposure to 1% serum and laminin, the spheres attached and cells rapidly migrated out onto the substrate. Clear differences in the total number of cells migrating were apparent in fields around neurospheres generated from each region (Fig. 4C). This was in large part due to the high proportion of small, phase bright immature neurons emerging from the  $^{\text{ctx}}$ NS and  $^{\text{str}}$ NS, which labelled with the neuronal marker TuJ1 (Fig. 4D,E).

Detailed analysis of the neuronal morphology revealed that cortical neurospheres gave rise to neurons that were significantly smaller than those from mesencephalic neurospheres (mean area of cortex-derived neurons  $\pm$  S.E.M. =  $64.5 \pm 1.59$ ,  $n=154$  cells; mesencephalon-derived neurons =  $137.6 \pm 6.00$ ,  $n=67$  cells; significantly different at  $P < 0.0001$ , Student's  $t$ -test). Furthermore, neurons from mesencephalic and cerebellar neurospheres often had long axonal process with characteristic blebs, not seen in neurons from cortical or striatal neurospheres (Fig. 4D, arrowheads), although very few cells from any region ( $< 0.01\%$ ) stained for tyrosine hydroxylase. Neurons from striatal neurospheres did not stain for choline acetyltransferase or dopamine and adenosine-related phosphoprotein (DARPP-32), but many were positive for GABA and glutamate as described previously [7] suggesting only selective neurochemical phenotypes were emerging from these neurospheres. We have recently shown that addition of growth factors further increases the number of cortically derived neurons [7]. In contrast, the small number of neurons generated from hind-brain neurospheres did not

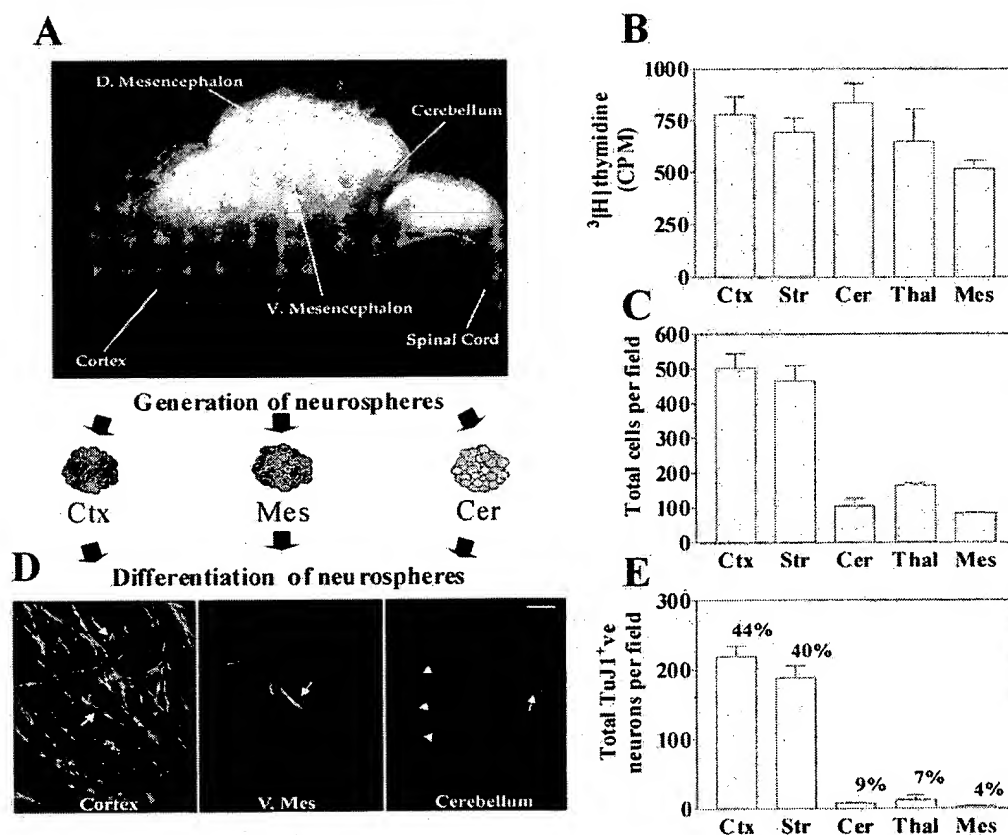


Fig. 4. Human neural precursor cells are regionally specified. (A) Brain tissue was removed from post mortem human fetal tissues (8–10 weeks post conception). (B) [ $^3\text{H}$ ]Thymidine uptake over 24 h by neurospheres derived from the cortex (ctx), striatum (str), cerebellum (cer), thalamus (thal) or mesencephalon (mes) showed similar rates of uptake, with the lowest being the  $^{\text{mes}}$ NS. (C) Total number of cells which migrated out from each region over 14 days. Note the marked differences in total migrating cells between the cortex and striatum compared with other regions. (D) Staining of the migrating cells from the different neurospheres with antibodies to TuJ1 (red; neuronal marker) and GFAP (green; astrocyte marker) revealed small neurons without long axonal processes from the striatum and cortex, but larger neurons with longer processes from other brain regions. Nuclei were labelled with Hoechst (blue). (E) The cortex and striatum were unique in generating large numbers of neurons.  $n=3-5$  separate foetal samples. Scale bar represents 50  $\mu\text{m}$ .

significantly increase upon growth factor addition, suggesting that trophic factor dependent cell death is not responsible for the differences in neuronal number (data not shown). In contrast to the rodent cultures described above, only a small number of oligodendrocytes were generated from early passage human neurospheres from every region used in this study (~1%), and the number of oligodendrocytes further declined to very low levels at later passages (<0.01%).

To determine how much cell division occurs following plating, and to establish the types of cells from <sup>ctx</sup>NS that give rise to the small Tuj1 positive neurons, we used time lapse cinematography to image the cells as they emerged from the sphere. At 3 days after plating a number of cells were seen to be dividing within the region around the sphere in the absence of either EGF or FGF-2. These cells were either very small, oval migratory cells or large more static cells with a type 1 astrocyte morphology. All of the divisions within this 2D environment were symmetrical (Fig. 5; see website for live image: <http://www.waisman.wisc.edu>). A large cell was never seen to give rise to a small cell and large cell upon division under these conditions (over 200 divisions followed for 24 h). Thus, the large number of neurons that emerge from the <sup>str</sup>NS or <sup>ctx</sup>NS could in part be due to division of small migratory neuronal progenitors post-plating, as described previously in the rodent system [23].

### 3.4. FACS analysis confirms regional differences within growing spheres

We next wanted to establish the nature of the dividing cells within the human neurospheres. Since we were unable to clone single cells from these cultures (they require continual cell–cell contact to divide under the present culture conditions), and our time lapse data did not show asymmetric divisions, we were not able to prove that a single precursor was dividing asymmetrically to generate both neurons and astrocytes. It was possible that two cells were dividing alongside each other: one a small neuronal progenitor (enriched in cortical primary tissue) and the other a large astrocyte progenitor (enriched in hind-brain primary tissue). To investigate this further, a method previously developed to distinguish various cell types within rodent neurospheres based on flow cytometry (FACS) was used [25]. FACS analysis of human neurospheres based on cell size and auto fluorescence revealed that there was a population of small, weakly fluorescent cells found only in neurospheres derived from the fore-brain (Fig. 6A, R3). Following differentiation for 7 days, the proportion of small cells increased dramatically in forebrain neurospheres (Fig. 6B, R3). In addition to the increase in this small cell population, the cells themselves were apparently smaller. Similar populations of small cells were absent from the <sup>mes</sup>NS (Fig. 6B, R3). These results

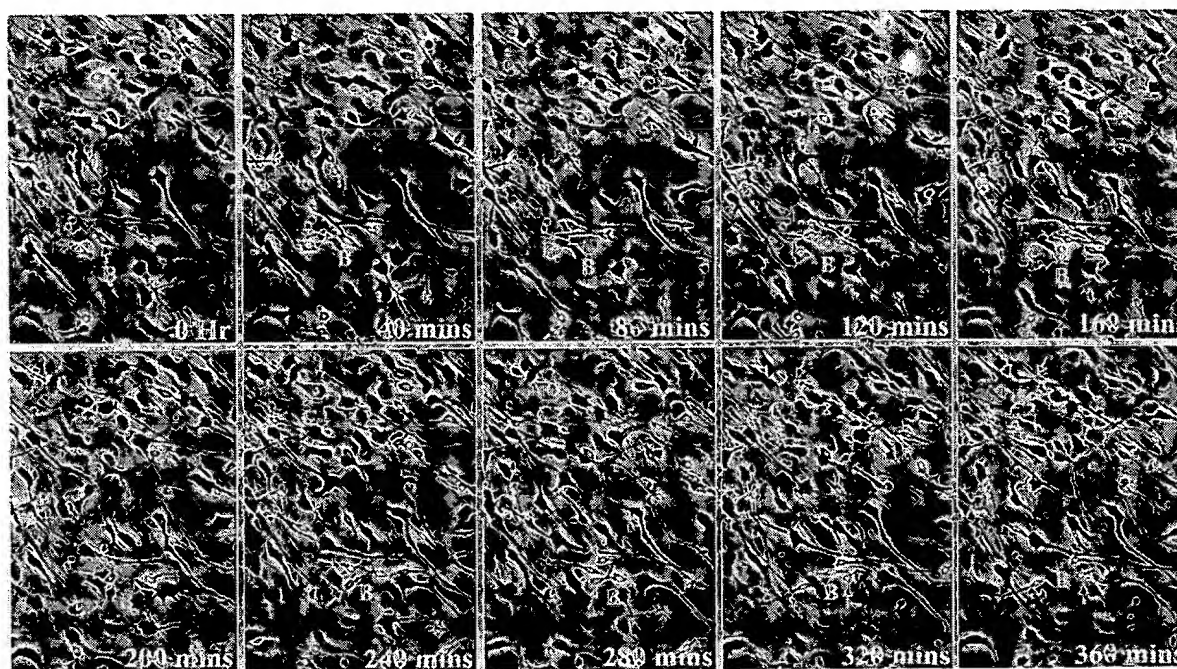


Fig. 5. Time lapse recording of cells around a human neurosphere dividing and migrating. Spheres were plated onto laminin in the absence of EGF and allowed to differentiate for 3 days. Interestingly, even in the absence of mitogens some division continued post plating. (A) Representation of a small migratory neuroblast which would invariably be Tuj1 positive (Fig. 7). (B) Representation of a small migrating neuroblast which underwent cell division (arrows) to give rise to two anatomically similar daughters which then migrated away. (C1 and C2) Representation of two large cells with an astrocytic morphology which underwent cell division (arrows) and gave rise to very similar large daughter cells. These large cells were far less migratory than the smaller ones. A video sequence can be seen in the on-line version of the paper, available on <http://www.neuroscion.com>.

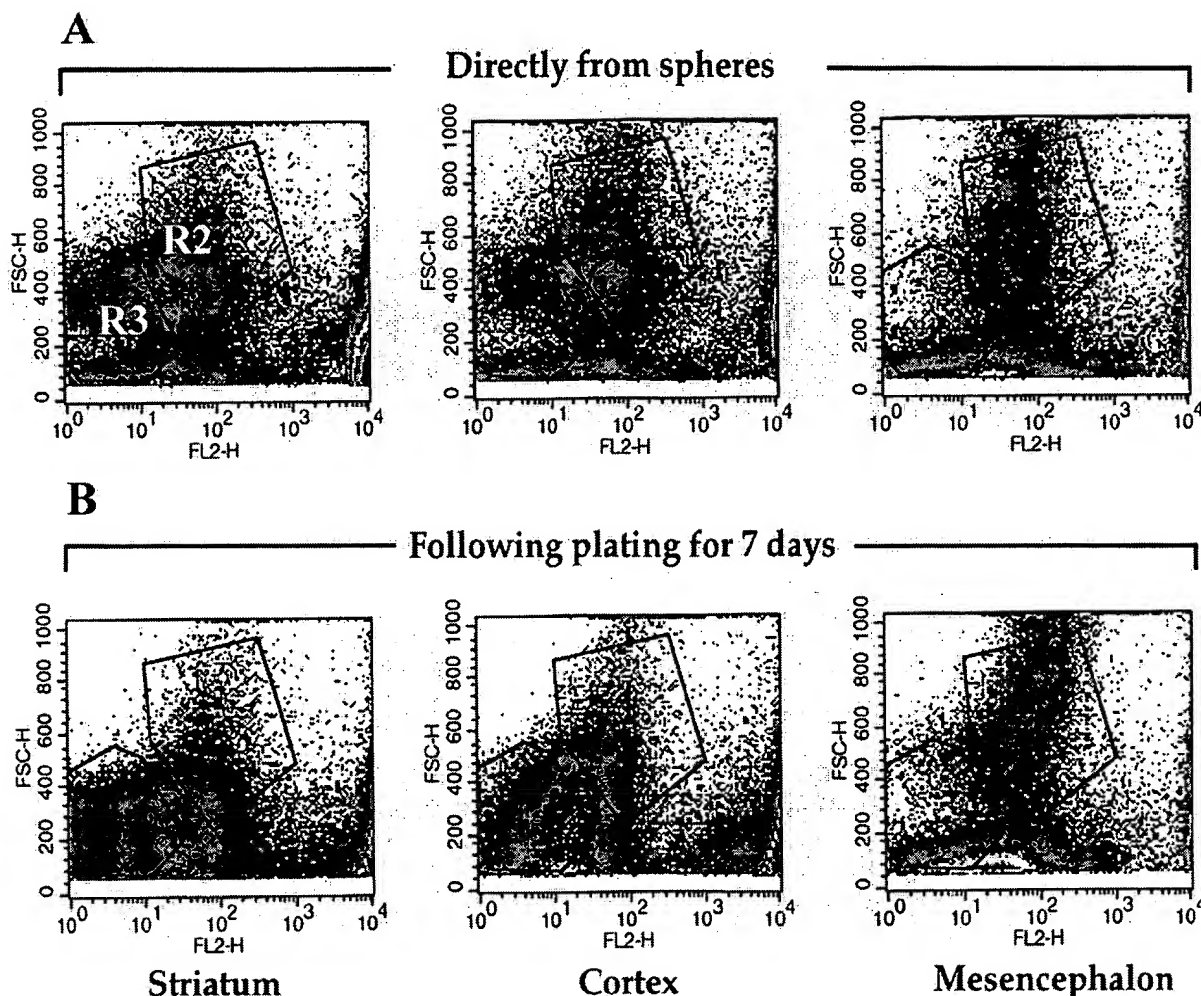


Fig. 6. FACS analysis confirms regional differences are apparent even within neurospheres. (A) Human neurospheres from different brain regions grown for 20 weeks as described in the Materials and methods section were dissociated and stained with propidium iodide, which labels dead cells, and sorted by size (Y-axis) and intensity of fluorescence (X-axis). Dead cells appear as highly fluorescent band to right of plot. Each dot represents a single cell. Neurospheres from the cortex and striatum showed a side band of smaller, less fluorescent cells (in region R3) which were missing in neurospheres from the mesencephalon. (B) Following differentiation for 7 days, the  $^{cix}$ NS and  $^{mNS}$  gave rise to many small cells which appear in R3. These were not seen to develop from the mesencephalon derived neurospheres. This analysis was repeated using three separate cultures with similar results.

therefore suggest that these cells represent the large number of small neuronal progenitors seen to arise from these cultures, and shown to be actively migrating in Fig. 5.

To determine whether the unique population of small cells in neurospheres derived from forebrain were indeed small self-renewing neuronal progenitors, cells from human  $^{cix}$ NS were sorted and separated into large (R2) and small (R3) populations (Fig. 7A). These cells were either plated onto laminin to assess differentiation, or seeded at high density with EGF to generate new neurospheres. After plating onto laminin, R2 cells showed a large amorphous phenotype, and a number of these had already begun to express GFAP after only 24 h (Fig. 7B). By 7 days nearly all of the cells in these cultures were GFAP positive (not shown). No  $TuJ1^{+ve}$  neurons were detected in these cultures at any time point. In contrast,

after plating onto laminin the small R3 cells were enriched for  $TuJ1^{+ve}$  neurons while still containing a few GFAP $^{+ve}$  astrocytes (Fig. 7B).

Did the large cells, which form mostly astrocytes and no neurons following plating onto laminin (presumably due to not undergoing asymmetric divisions), have the potential to generate neurons? To test this we attempted to make new neurospheres from the sorted cell populations. Following re-exposure to EGF immediately following plating onto non-coated plastic dishes, the small R3 cells failed to generate any new neurospheres (Fig. 7C). In contrast, the large R2 cells plated at high density in the presence of EGF rapidly aggregated and formed new spheres (Fig. 7C). Following 14 days of growth, intact spheres derived from the large R2 group were re-plated as whole spheres onto laminin and allowed to differentiate. Within 7 days they had generated large numbers of small  $TuJ1^{+ve}$

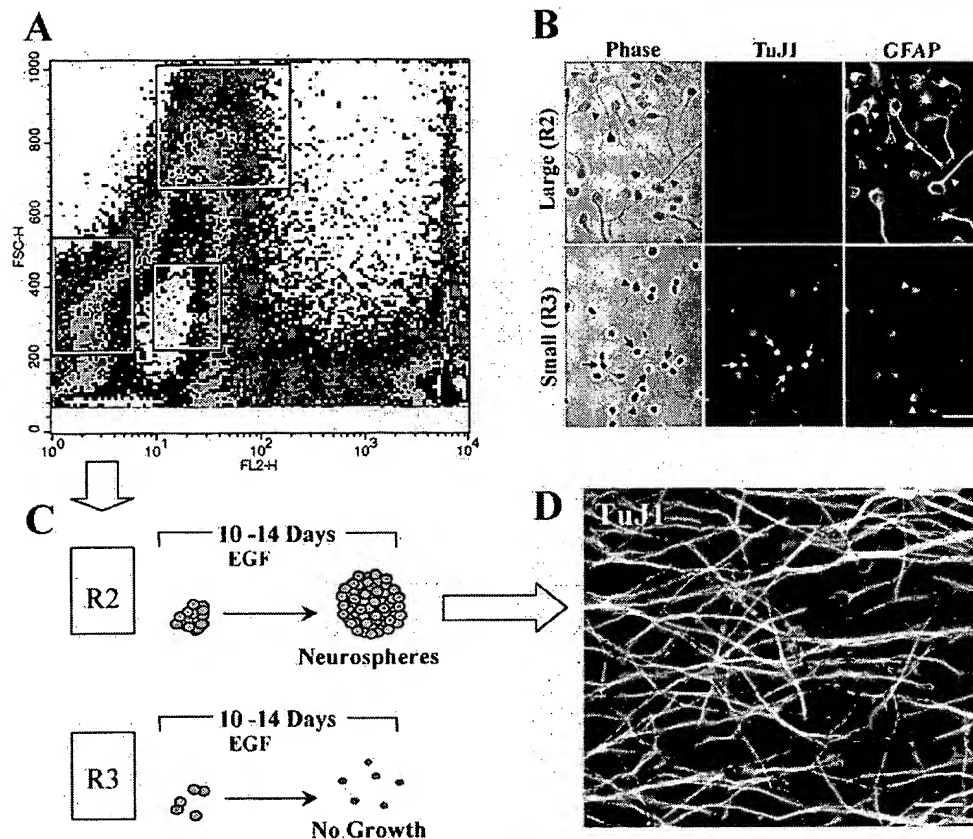


Fig. 7. A large cell with a glial morphology can give rise to neurons. (A) Neurospheres generated from the human cortex and grown for 20 weeks were sorted by FACS and two regions collected (large R2 and small R3). (B) Following acute plating (2 h) onto coated wells, the small R3 cells gave rise to a high numbers of TuJ1 positive neuroblasts (arrows) with a few GFAP positive cells (arrowheads). In contrast the large R2 group gave rise to both GFAP (arrowheads) and unlabelled cells but no TuJ1 positive neuroblasts. Scale bar represents 80  $\mu$ m. (C) Following re-exposure to EGF at high plating densities the R2 group generated new neurospheres whereas the R3 group did not respond to EGF. (D) Following 2 weeks of expansion, neurospheres generated from the R3 group again generated large numbers of small TuJ1 positive neurons following plating for 7 days. Scale bar represents 50  $\mu$ m. This analysis was repeated twice with two separate cultures with similar results.

neurons (Fig. 7D). This demonstrates that the large cell population within the <sup>ctx</sup>NS is the neurosphere forming cell. Furthermore, this cell is able to generate a new population of small neuronal progenitors within the 3D environment of the neurospheres.

### 3.5. Discussion

#### 3.5.1. Limited growth of rat but not human neurospheres

In this study, and in our previous report [44], we were unable to grow rat <sup>stx</sup>NS for more than 6 weeks. Various techniques shown to enhance the growth of rat neurospheres, such as maintaining cell–cell contact, adding heparin to the medium, combining EGF and FGF or adding LIF to the medium (reviewed in Ref. [41]) did not overcome this lack of growth in the current study. The growth restriction was not simply a function of embryonic age, as a similar cessation in growth occurs in neuro-

spheres derived from tissue between E12 and P1 (Rossor and Svendsen, unpublished observations). Neurospheres generated from other brain regions also stopped growing after a short period of expansion. Paradoxically, rodent neurospheres express high levels of telomerase and have long telomeres [29], suggesting that they have enormous potential for growth. Human neurospheres, on the other hand, express only small amounts of telomerase and have shorter telomeres, yet grow for much longer periods [29]. Clearly in neurospheres derived from different species, there are cell proliferation checkpoint mechanisms that are independent of telomerase activity. Furthermore, it is likely that the culture conditions for rat neurosphere growth have not yet been optimised, and that their early cessation of proliferation could be overcome in the future. Indeed, rodent oligodendrocyte precursor cells, which normally can only undergo eight divisions in culture, can continue to divide for extended periods of time providing thyroid hormone is removed from the media [46]. We look forward to future improvements in the growth of rat neurospheres as well.

### 3.5.2. Regional specification of neurospheres in the absence of environmental signals

Classical retroviral studies have shown that at early stages of development, at least some single labelled cells can give rise to multiple types of neuron and glia [37]. Thus, there is widespread agreement that true multipotent neural stem cells exist *in vivo*. However, similar studies have also shown that within the developing cortex, many cells are more restricted progenitors, such as those giving rise to either pyramidal or non-pyramidal neurons [30]. Furthermore, cortical and striatal progenitors retain the capacity to differentiate into specific phenotypes, even when removed from their *in vivo* environment and induced to divide several times *in vitro* [14,38]. The fate of cortical neurons appears to be critically dependent on the signals the cells receive in the final stages of cell division [24]. In the neurosphere model used in the present study, the environmental cues are limited to the surrounding cells, all undergoing synchronous differentiation. These surroundings may be very different to the *in vivo* situation where polarity, growth factor gradients and a temporally defined range of different cells are influencing cell fate. Under the culture conditions employed here, all neurospheres gave rise to differentiating neurons, although the number and morphology were very different. Since at least some of these neurons had arisen from dividing neural precursors,

as indicated by BrdU co-labelling, the regional differences in neuronal emergence are most unlikely to reflect differences amongst post-migratory neurons remaining from the primary culture. Rather, they are likely to reflect the presence of regionally-specified cell-autonomous signals retained within neurospheres.

The migrating neuroblasts from the <sup>mes</sup>NS were consistently larger than those from the <sup>str</sup>NS or <sup>ctx</sup>NS and often had long axonal projection fibres. We propose that in the absence of other signals, there must be a program within the dividing and migrating progenitor cells which determines cell size and differentiation. As such they might be considered lineage restricted, perhaps in a similar fashion to the long-term and transiently self renewing populations of hematopoietic stem cells described previously [26]. Alternatively, the surrounding migrating glial cells may release factors that affect the size and number of migrating neuroblasts. It of course remains possible that under different culture conditions, or following grafting, the fate of these progenitor cells may be changed, i.e. they are not determined, but specified in the absence of other signals. In addition, there may be a few true multipotent, or even pluripotent stem cells in neurospheres from each brain region dividing slowly alongside more rapidly proliferating restricted neural progenitors (Fig. 8). These would be missed when large numbers of cells are plated simul-

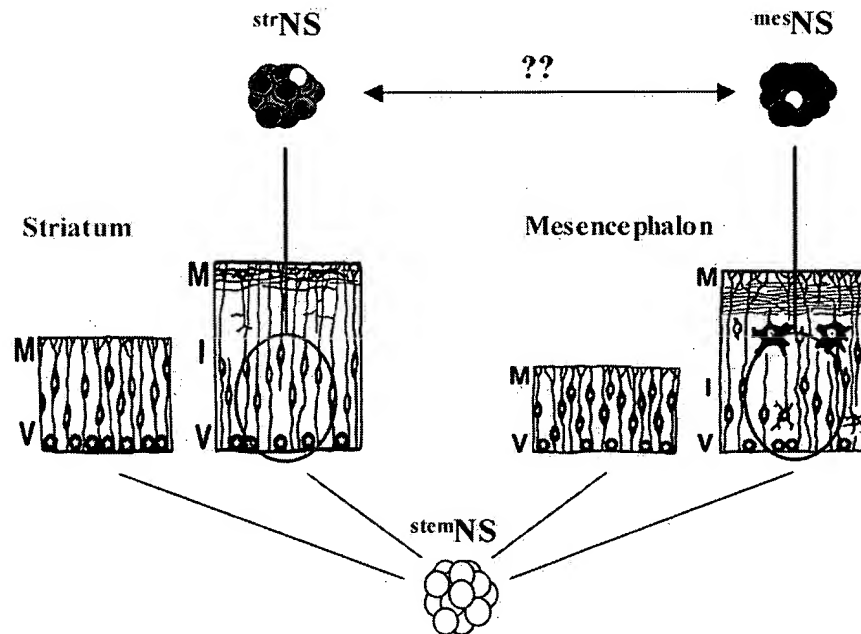


Fig. 8. Neurospheres are regionally specified. Our initial prediction was that perhaps a common cell could be isolated from all regions of the developing brain and grown into a neurosphere (<sup>stem</sup>NS in white). Instead, the majority of cells within the spheres had regional specificity, even after extended passaging. Those from the striatum (red) had a greater capacity to migrate out from the sphere and form neurons when compared to those from the mesencephalon (green). Our data do not exclude the possibility of non-specified, equipotent stem cells existing within spheres from both regions in very small numbers (white), which exist in ventricular zones (V) throughout the developing neuroaxis. This cell may be masked by the many specified cells. Nor do we know whether under different environmental conditions the fate of spheres from each region might be altered (dotted line). However, we hypothesize that the majority of cells within neurospheres arise from regionally specified progenitors within the intermediate zone (I). Further studies are now required to establish the mechanisms underlying these observations.

taneously. It is possible that these pluripotent stem cells may be responsible for the reported trans-differentiation of human neurosphere derived cells into muscle cells at low efficiency [18].

It is tempting to speculate that the relatively high proportion of neurons arising from rat <sup>str</sup>NS reflects the protracted period of neurogenesis in the rat striatum, extending from embryonic day 12 (E12) to E22 with a peak at E15/E16 [15]. In comparison, cortical neurogenesis in the rat occurs during a more restricted 6-day period from E13.5 to E19.5 [4]. Similarly, the neurons of the rat substantia nigra are generated from dividing precursor cells in the ventral mesencephalon over only 4–5 days, between E11 and E15 [1,19,21]. These features of neural development are consistent with the present findings that the <sup>str</sup>NS gave rise to more neurons than those from either <sup>mes</sup>NS or <sup>ctx</sup>NS. Thus, the rat neurosphere culture model may more closely resemble *in vivo* neural development than had previously been realized. The human neurosphere cultures were also regionally specified, but had a far greater capacity for division. Human neural development takes place over at least 9 months with complex patterns of division and migration for each brain structure. It is possible that the protracted division compared to the rat also represents a greater capacity for human cells to divide *in vitro* and produce the greater quantity of neurons found in the human brain compared to the rodent. We are currently further studying mechanisms of continual proliferation in these human neurosphere cultures.

It was notable that, despite the novel passaging techniques used here for optimising cell–cell contact between proliferating NPCs, the <sup>mes</sup>NS only gave rise to very small numbers of TH-positive neurons in comparison to primary mesencephalon cultures. This is consistent with the data suggesting that embryonic dopamine neuroblasts have a limited capacity to undergo *in vitro* proliferation and expansion [6,8,39]. Indeed, it is recognised that the effective *in vitro* expansion and/or induction of the DA phenotype in propagated NPCs is likely to require their genetic modification in combination with the appropriate extrinsic inductive signals [50]. Therefore, while there are clear regional differences in the numbers and size of neurons, the generation of neurochemical phenotypes specific to each region is likely to require additional signals which are not present in our culture system. It is of interest that the human cerebellar and thalamic derived neurospheres also generated low numbers of neurons. Based purely on developmental maturation, the cerebellum might be expected to contain the most primitive type of stem cell, and to retain the capacity to produce high numbers of neurons due to the enormous volume of cerebellar granular cells found in the mature human brain. However, this was not the case. It might be that there is an optimal developmental window for harvesting human neural cells capable of making neurospheres with a high

neuronal differentiation potential, and that this may differ between brain regions. We are currently investigating whether this is in fact the case.

### *3.5.3. The special capacity of forebrain neurospheres to generate high numbers of neurons*

Why should the neurospheres derived from the human forebrain produce such high numbers of small neurons even after extensive passaging, when the mesencephalon, thalamus and cerebellum produced relatively few? The sub-ventricular zone (SVZ) of the adult rodent forebrain has been shown to harbour cells expressing astrocyte markers (type B cells) which are capable of producing neuronal progenitors (type A cells) via an intermediate cell (type C cell [13]). The type A neuronal progenitors then migrate along the rostral migratory pathway into the olfactory bulb [22]. This three cell system, where the type C cell is capable of generating large numbers of small migrating neurons, is not only unique to the mammalian forebrain, but is also absent in hindbrain structures which do not have an SVZ or in avian species. A very similar three cell system has recently been described in the primate forebrain [20]. We have shown through FACS analysis that a large cell within the forebrain neurospheres, which upon acute differentiation and plating exclusively generated astrocytes, was able to generate neurons when re-exposed to EGF and expanded again in neurosphere cultures. It is possible that this cell may be a type B cell, which within the three dimensional environment of the neurosphere is able to generate type C cells that in turn produce small type A neuronal progenitors. This cell may not be present in neurospheres derived from other brain regions, which were only able to generate neurons directly from the type B cells with no type C intermediate. Although this hypothesis remains highly speculative, it is worthy of further investigation and provides at least one potential mechanism by which the cortical and striatal human cultures are able to maintain such a high neuronal output.

We show here that neurospheres generated from all regions of the human brain were able to produce both astrocytes and neurons, but very few oligodendrocytes at later passages. The lack of oligodendrocyte production seen here and by others at late passages using similar culture systems [10,48] suggests perhaps that either (i) the methods used to grow long-term neurosphere cultures favour committed neuron/astrocyte progenitors rather than multipotent neural stem cells or (ii) they are all multipotent stem cells, but the culture conditions are not correct to produce oligodendrocytes. It is very difficult to distinguish between these two possibilities until we understand more about these cells. However, there is again a clear species difference in that the rodent cultures described produce many oligodendrocytes even if generated from regions of the spinal cord not expected to produce them [11]. A lack of oligodendrocytes does not imply that they could not be

generated from these human cultures under the correct culture conditions, or following transplantation. However, we have so far failed to achieve this effect using a range of different paradigms (Chandran and Svendsen, unpublished observations).

### 3.5.4. Implications for cell therapy using human neural stem cells

The data presented here suggest that there are significant regional differences when neurospheres are derived from different areas of the developing rodent or human brain. Thus, many of the cells dividing within neurospheres retain a memory of their origin. This may be a result of the different developmental stages the regions were at when cultured, or simply that each neurogenic zone of the developing brain has a specific type of cell which responds to EGF and FGF-2. Regardless of the mechanism underlying this phenomenon, it will be important for transplantation studies using cells derived from human neurospheres to establish their exact origin. It is possible that those from regions other than the striatum or cortex are not likely to generate large numbers of neurons following transplantation, although the effects of different environmental signals now need to be investigated. Hindbrain neurospheres produce less neurons, but those that were generated were larger and had longer processes. Thus we speculate that while cortical/striatal neurospheres may be useful for replacing lost inter-neurons, hindbrain neurospheres may be better suited to replacing large projection neurons. However, this remains to be established in transplantation models.

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